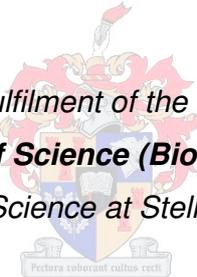


Cloning and functional characterization of thiol disulfide interchange system proteins from *Staphylococcus aureus*

by
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Thesis presented in partial fulfilment of the requirements for the degree of
Master of Science (Biochemistry)
in the Faculty of Science at Stellenbosch University



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Declaration

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Summary

The 21st century has seen the re-emergence of infectious diseases thought previously conquered by antibiotics. The human pathogen, *Staphylococcus aureus*, is one such example, with global reports listing resistance to antibiotics from methicillin to vancomycin, the so-called “drug of last resort”. The speed at which such pathogens acquire antibiotic resistance has led to the questioning of the current strategy of developing drugs that kill infectious agents directly. Instead, new tactics must be employed that rely more heavily on the host’s own immune defences. *S. aureus* circumvents the human immune system’s oxidative killing mechanisms through the actions of a thiol-disulfide interchange system which maintains intracellular redox balance within the pathogen. Disruption of the system and its players thus represents a potential target for the development of novel anti-staphylococcal agents. This study set out to characterise three proteins suggested to be involved in the thiol-disulfide interchange system so as to assess their roles and their viability as effective drug targets.

This study had two major aims: the functional characterisation of the MerA and YpdA flavin disulfide reductase homologs, and the assessment of the substrate promiscuity of the TrxB and TrxA proteins. The characterisation of MerA and YpdA involved activity assays with the LMW thiol disulfide substrates involved in *S. aureus* redox balance. While failing to show demonstrable disulfide reductase activity, MerA instead was found to have the ability to reduce two biologically-relevant transition metal ions without the aid of an interacting protein partner: Hg²⁺ and Fe³⁺. Fe³⁺ was further shown to be favoured over Hg²⁺ as a substrate of MerA, which has implications for the protein’s role in oxidative stress resistance. YpdA was shown to be incapable of reducing any of the LMW thiol disulfides found in *S. aureus*, even in the presence of a generic TFP protein, TrxA. This suggests that YpdA may be unable to perform catalytic functions in the absence of its correct interacting partner. Alternatively, the protein may perform a different cellular function unrelated to LMW thiol disulfide reduction or oxidative stress resistance.

The substrate promiscuity of the thioredoxin system proteins of *S. aureus*, TrxB and TrxA, was demonstrated through their measurable reduction of three LMW thiol disulfides. However, the poor kinetic parameters determined for the reactions indicate that these proteins may be more likely to reduce the LMW thiol disulfides under critical conditions, as opposed to acting as their primary reducing system. Importantly, this study has demonstrated a role in the maintenance of *S. aureus* redox balance beyond the usual cellular functions of TrxB and TrxA. These findings provide additional support for TrxB as a viable target for the development of novel antibiotics.

Opsomming

Die 21ste eeu het die terugkeer gesien van aansteeklike siektes wat voorheen deur antibiotika beheer kon word. Die menslike patogeen, *Staphylococcus aureus*, is een so 'n voorbeeld, met internasionale verslae wat weerstandigheid lys teen geneesmiddels van methicillin tot vancomycin, die sogenaamde "geneesmiddel van laaste uitweg". Die spoed waarteen sulke patogene weerstand ontwikkel teen antibiotika lei tot die bevreemding van die huidige strategie van geneesmiddels ontwikkel om bakterieë te dood. In teenstelling word nuwe taktiek benodig wat eerder staatmaak op die mens se eie immuunstelsel. *S. aureus* vermy die menslike immuunstelsel se oksidatiewe verdedigingsmeganismes deur die gebruik van 'n tiol-disulfied uitruilingstelsel wat intrasellulêre redoksbalans beheer binne die patogeen. Ontwrigting van die stelsel verteenwoordig dus 'n potensiële teiken vir die ontwikkeling van nuwe antistafilokokkale agente. Die doel van hierdie studie was om drie proteïene te karakteriseer wat betrokke is in die tiol-disulfied uitruilingstelsel, om sodoende hulle rolle te assesseer en hulle lewensvatbaarheid as teikens vir antibiotika te evalueer.

Hierdie studie het twee hoofdoelwitte gehad: die funksionele karakterisering van die MerA en YpdA flavoproteïene, en die assessering van substraat-promiskuiteit van die TrxB en TrxA proteïene. Die karakterisering van MerA en YpdA het bestaan uit die uitvoering van aktiwiteitstoetse met die tiol disulfide substrate wat betrokke is in die handhawing van *S. aureus* se redoksbalans. Hoewel MerA geen bewese disulfide reductase aktiwiteit gedemonstreer het nie, het die proteïen eerder die vermoë gedemonstreer om twee biologies-relevante metaal ione te reduceer sonder die hulp van 'n interaktiewe proteïen-vennoot: Hg^{2+} en Fe^{3+} . Fe^{3+} is verkies bo Hg^{2+} as 'n substraat van MerA, wat implikasies het vir die proteïen se rol in oksidatiewe stress-weerstand. Daar is bewys dat die YpdA proteïen geen van die tiol disulfiedes kon reduceer nie, selfs in die teenwoordigheid van 'n generiese TFP proteïen, TrxA. Dit dui daarop dat YpdA nie in staat mag wees om katalitiese funksies uit te voer nie, in die afwesigheid van die korrekte interaksie vennoot.

Die substraat-promiskuiteit van die thioredoxin-sisteem proteïene van *S. aureus*, TrxB en TrxA, is gedemonstreer deur die meetbare redusering van drie tiol disulfied substrate. Tog dui die swak kinetiese parameters vir hierdie spesifieke reaksies daarop dat hierdie proteïene nie as primêre reduseeringsstelsel vir hierdie verbindings op te tree nie. Hierdie studie het 'n rol vir TrxB en TrxA in die handhawing van *S. aureus* redoksbalans gedemonstreer wat buite die proteïene se gewone sellulêre funksies is. Hierdie bevinding gee addisionele ondersteuning aan TrxB as 'n doeltreffende teiken vir die ontwikkeling van nuwe antibiotika.

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“If the world is full of darkness, make it your business to build fires”

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Abbreviations

AMP	Adenine monophosphate
Arg	Arginine
ATP	Adenine triphosphate
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
BSH	Bacillithiol
BSSB	Bacillithiol disulfide
CoA	Coenzyme A
CoAD	Coenzyme A disulfide
CoADR	Coenzyme A disulfide reductase
Cys	Cysteine
ddH ₂ O	Distilled, deionised water
DNA	Deoxyribonucleic acid
DTNB	Ellman's reagent
EDTA	Ethylenediaminetetraacetic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EtOH	Ethanol
FAD	Flavin adenine dinucleotide
FDR	Flavoprotein disulfide reductase
GSH	Glutathione
GR	Glutathione disulfide reductase
H ₂ O ₂	Hydrogen peroxide
His-tag	6xHistidine tag
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl β -D-1-thiogalactopyranoside
k_{cat}	Turnover number
KDa	Kilodalton
K _M	Michaelis constant
LB	Luria Bertani
LMW	Low molecular weight
<i>M. tuberculosis</i>	<i>Mycobacterium tuberculosis</i>
MeOH	Methanol
Mn	Manganese
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>

NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NOX	NADPH oxidase
O_2^-	Superoxide anion
OD ₆₀₀	Optical density at 600nm
OH ⁻	Hydroxyl anion
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TFP	Thioredoxin fold protein
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
Trx	Thioredoxin
TrxR	Thioredoxin reductase
V_{max}	Maximal velocity

Chapter 1

Introduction to *Staphylococcus aureus* and the human immune system

1.1 *Staphylococcus aureus* as a human pathogen

Staphylococcus aureus is a pathogen primarily responsible for most purulent skin and soft tissue infections in humans. It is the main causative agent for a range of diseases, including epidermal abscesses, endocarditis and toxic shock syndrome.^{5, 6} Although the bacterium is commonly found on the skin and mucosal surfaces of 25-35% of healthy human beings¹⁵, it remains an opportunistic pathogen; *S. aureus* may invade and infect tissues wherever epithelial integrity is weakened, be it from injury or within the confines of medical procedures. As a result of this, *S. aureus* is a leading cause of both nosocomial and community-acquired infections.¹⁴

1.1.2 The health threat posed by drug-resistant *S. aureus*

The rising incidence of drug resistance within disease-causing bacteria is a worrisome threat. The 21st century has seen the rise of both new infectious diseases and the re-emergence of those thought previously conquered by antibiotics, such as tuberculosis.¹² *S. aureus* is no exception to the trend, having displayed formidable ability in acquiring resistance to multiple antimicrobial agents. The pathogen's speedy evolution is apparent: in 1946, *S. aureus* infections were readily treated with penicillin. Today however, more than 50% of all *S. aureus* infections reported in the U.S.A are methicillin-resistant.¹⁵ Furthermore, many display additional resistance to second-line antibiotics, such as clindamycin and trimethoprim-sulfamethoxazole.^{4, 11} Most recently, reports of strains found to have resistance toward vancomycin, the so-called "drug of last resort", have been on the rise worldwide.^{8, 15} The incidence of methicillin-resistant *S. aureus* (MRSA) is also troublingly common in many countries across the globe, ranging from 10% - 50% of the total reported *S. aureus* infections (Figure 1.1). The evidence provided in these statistics is clear: utilising antibiotics that directly kill pathogens does not work in the long-term. Rather, this strategy promotes antibiotic resistance and thus creates pathogens that are more formidable. In order to combat this rise in drug resistance, new tactics must be employed that rely more heavily on

the host's own immune defences. New antibiotics should thus weaken the bacterium, or alternatively strengthen the host immune system so that it may handle the infection alone.

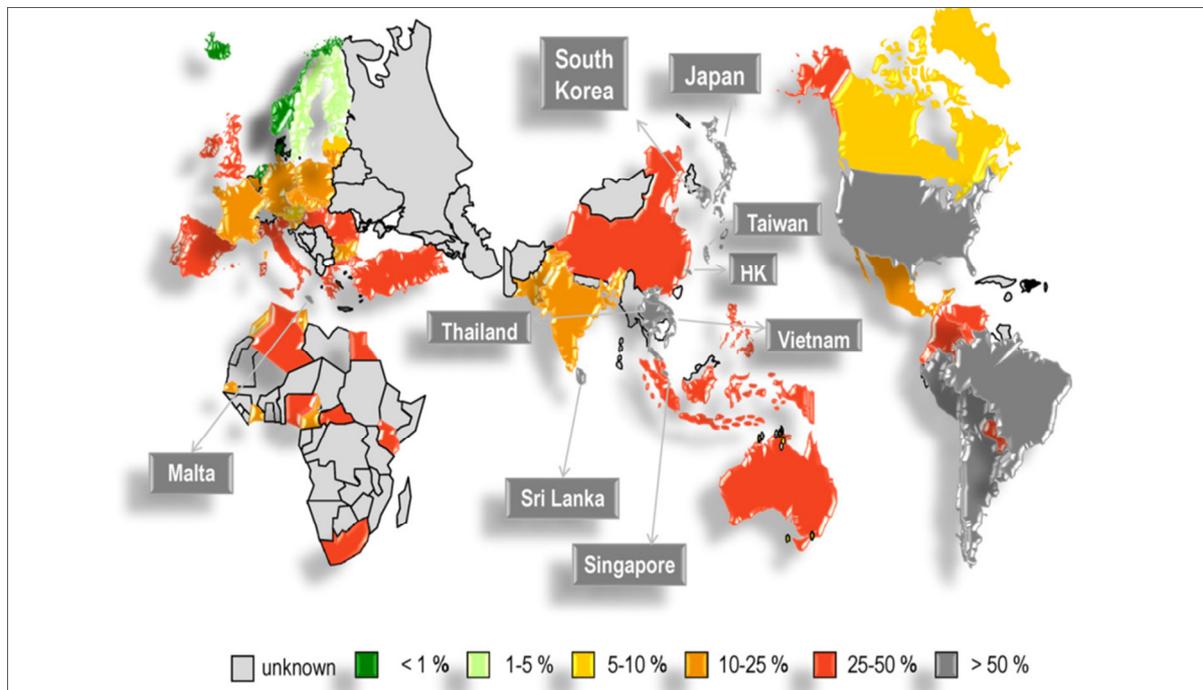


Figure 1.1: Global prevalence of hospital acquired MRSA infections in 2012¹⁵

1.2 Reactive oxygen species and their role in the host immune response

Despite its necessity to and use in many metabolic processes, oxygen is a highly reactive molecule. Partial reduction of oxygen leads to the formation of reactive oxygen species (ROS), most notably hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$) and hydroxyl radicals (OH^{\cdot}).⁷

These reactive species can cause extensive damage to proteins, lipids and DNA if not dealt with, and as a result of this, all aerobic organisms are equipped with various systems for neutralising and eliminating moderate exposure to ROS. The human innate immune system takes advantage of oxygen's toxicity, directly attacking pathogens with a blast of ROS in order to eliminate them from the host.^{5, 16} Bacterial pathogens must therefore be capable of surviving two distinct sources of ROS: those generated through normal cellular metabolism, and those used by immune defences as a form of attack.

1.2.1 Sources of ROS

1.2.1.1 ROS from the electron transport chain

The electron transport chain (ETC) in bacteria is used to produce ATP via the movement of electrons. Electrons obtained from metabolic components, such as NADH and FADH₂, are channelled through four membrane-embedded complexes, with O₂ serving as the final electron acceptor. The energy that is released through electron movement down a reduction potential is used to create the H⁺ gradient that drives ATP production.¹³

Imperfections in the system however, lead to the minor production of O₂^{•-}. Electrons are capable of leaking into the periplasmic space from complexes I and III of the ETC, at which point they will reduce the O₂ present there (Figure 1.2A). The ETC alone is responsible for converting up to 2% of the oxygen consumed by the cell into O₂^{•-}. In order to mitigate any oxidative damage, any O₂^{•-} produced is neutralised by superoxide dismutase (see chapter 2).¹³

1.2.1.2 ROS from the NADPH-oxidase complex

When an infection is detected within the host, cytokines are released by the inflamed epithelium, thus drawing phagocytic macrophages and neutrophils to the site.¹⁶ Consequently, the pathogen will be engulfed by these cells and subjected to a combination of both oxidative and non-oxidative killing mechanisms. The former relies on the generation of ROS by a phagocytic NADPH-oxidase complex (NOX), whilst the non-oxidative methods include the use of degradative proteases.^{1, 16}

While the neutrophil is at rest, most NOX complexes are located on the membranes of intracellular granules, with only 10-20% found on the cellular plasma membrane.¹⁷ Upon recruitment of the neutrophil to the infection site, granules will fuse with the phagosome membrane encasing the pathogen, thus further enriching the membrane with NOX complexes. Prior to O₂^{•-} generation, NOX must be activated through the association of a FAD cofactor to the enzyme.¹³ Various NOX complexes exist within neutrophils, each with different cell or tissue-specific expression patterns dictating a particular enzymatic core (NOX 1-5, Duox 1 or Duox 2). Different complexes may additionally require particular cofactors for activation, such as bound calcium or specific proteins (Figure 1.2B).^{1, 13}

NOX assembled on the phagosome generates O₂^{•-} by channelling electrons acquired from cytoplasmic NADPH through complex-bound FAD and two heme *b* prosthetic groups.¹³ The electrons will move across the phagosomal membrane via the above-mentioned electron

carries, finally passing from heme to molecular oxygen, creating $O_2^{\cdot-}$. The $O_2^{\cdot-}$ may be further dismutated (either spontaneously or via SOD) to form H_2O_2 and molecular oxygen.^{13, 19} Additionally, any H_2O_2 and $O_2^{\cdot-}$ generated by NOX complexes at the neutrophil plasma membrane may move across the phagosome (via diffusion or anion channels respectively) in order to attack the bacterial target.^{13, 17}

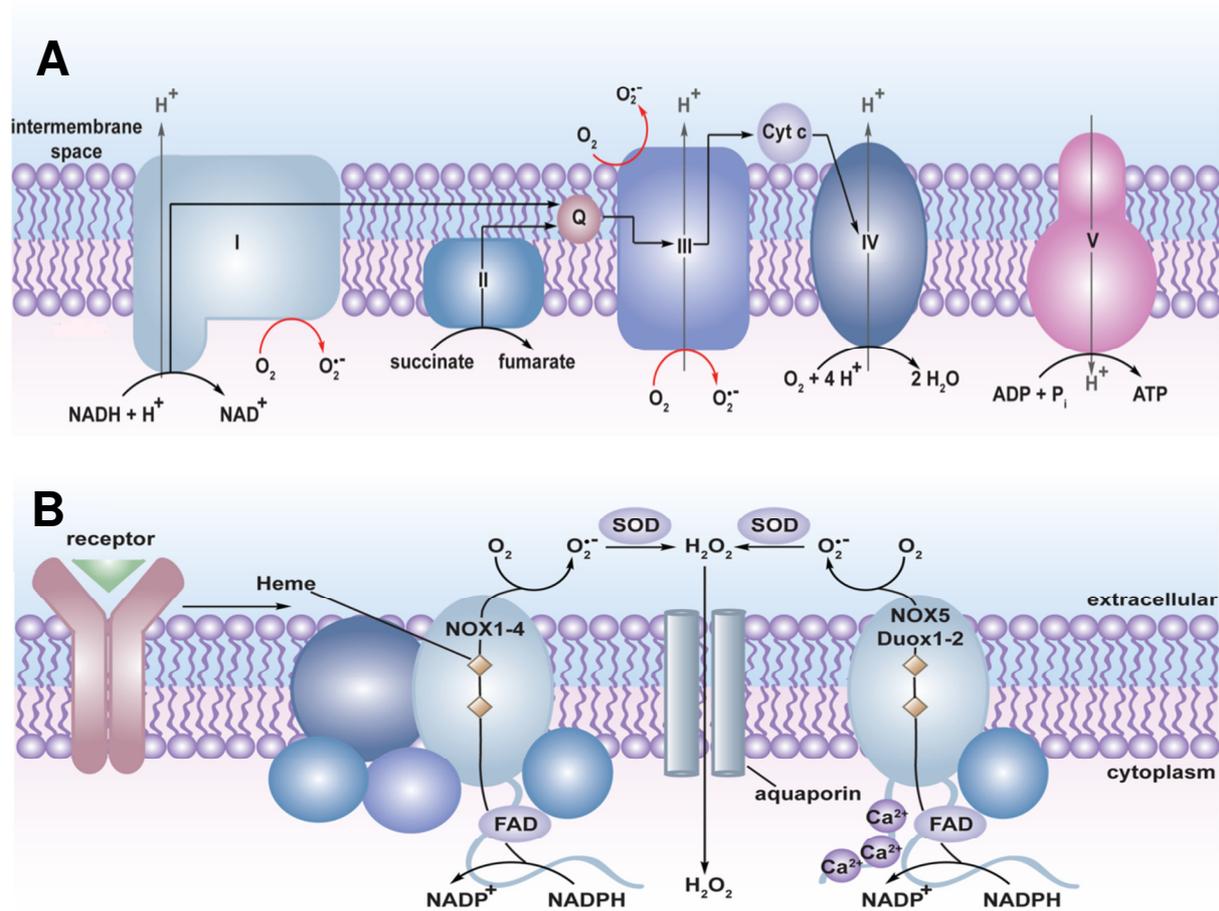


Figure 1.2: The sources of ROS. **A)** The electron transport chain (illustrated here for mitochondria) converts up to 2% of the oxygen consumed by the cell into $O_2^{\cdot-}$. This occurs when electrons leak from complexes I and III into the intermembrane space outside of the cell membrane. The electrons will then be used to reduce O_2 to $O_2^{\cdot-}$. **B)** Activated NADPH oxidase assembles at both the neutrophil cell membrane (pictured here) and the phagosome membrane to generate $O_2^{\cdot-}$. Electrons from cytoplasmic NADPH are donated to the FAD moiety of the complex and are passed through two heme groups before reducing O_2 . A portion of the $O_2^{\cdot-}$ generated may then be further dismutated to H_2O_2 . Figure reproduced from Paulsen et al.¹³

1.2.2 Other oxidants

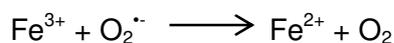
In addition to ROS, phagosomes are capable of producing hypohalous acids (HOX) that are further detrimental to invading pathogens. HOXs may be produced through the reaction of a halide ion (Cl^- , Br^- or I^-) with H_2O_2 , which is catalysed by the enzyme myeloid peroxidase.^{13, 16} The oxidation of any particular halide ion results in the formation of its corresponding HOX, e.g. hypochlorous acid (HOCl) is produced through the oxidation of Cl^- with H_2O_2 .¹³

HOXs are potent bactericidal compounds, readily oxidising any thiol groups or methionine residues they come into contact with. In addition to this, HOCl specifically may react with $\text{O}_2^{\cdot-}$ to produce OH^\cdot . This is extremely problematic for the bacterial cell, as OH^\cdot is a powerful radical to which the cell has no specific enzyme antioxidants.^{13, 16}

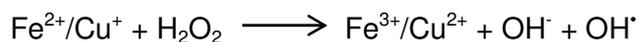
1.2.3 Fenton chemistry and ROS

Within bacterial cells, H_2O_2 and $\text{O}_2^{\cdot-}$ may react with various trace metal ions to produce OH^\cdot as a result of the Haber-Weiss reaction and Fenton chemistry as follows:¹⁸

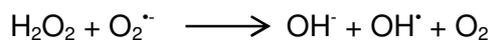
Haber-Weiss Reaction:



Fenton Reaction:



Net reaction:



Although this phenomenon is not directly utilised by the immune system, it is nonetheless important for its added detrimental effect on the pathogen. Thus the neutrophil need only generate H_2O_2 and $\text{O}_2^{\cdot-}$, whilst the natural chemical reactions between these ROS and trace metals within the bacteria will lead to the additional production of OH^\cdot .

1.2.4 The effects of ROS and oxidants on the bacterial cell

The ROS and other oxidants produced by the neutrophil will proceed to oxidise lipids, DNA and, critically, the thiol groups of any cysteine residues found in the proteins of bacterial pathogens.^{3, 9, 16} The oxidation of these thiol groups leads to the formation of sulfenic acids and, if allowed to continue, the original thiols will be irreversibly oxidised to sulfinic and

sulfonic acids (Figure 1.3).^{3, 19} This creates irreparable damage to the proteins essential to the pathogen's survival. In combination, these processes all contribute to the toxic effects of oxidative stress that will cumulatively cause the bacteria to die if they do not employ mechanisms to resist these effects.

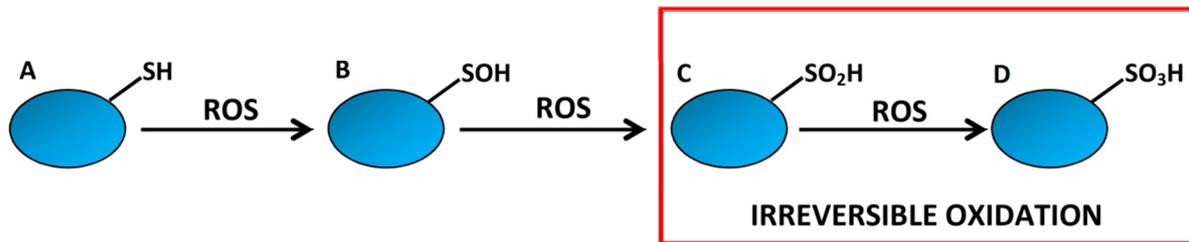


Figure 1.3: The effects of ROS exposure on protein thiol groups. Initial exposure of reduced protein thiol groups (A) to ROS leads to the formation of a sulfenic acid (B). Sulfenic acid may still be converted back to an ordinary thiol group. However, prolonged exposure to ROS leads to irreversible oxidation through the formation of sulfinic and sulfonic acids (C and D respectively).

1.3 Focus of this study

S. aureus, like many other organisms, employ several specific mechanisms to counter oxidative stress. However, in its case these mechanisms are even more crucial as it ensures the survival of these bacteria within the human host by naturally countering the oxidative killing mechanisms of the human immune system. As such, these mechanisms can be considered as attractive targets for the development of novel drugs; by disabling the system, *S. aureus* would be made more vulnerable to destruction by the body's own immune response. However, we do not yet understand the exact mechanisms and components involved in these resistance mechanisms well enough in order to exploit it.

1.3.1 Aim and objectives of this study

The two main aims of this study was to 1) functionally characterize the putative flavin disulfide reductase (FDR) enzymes MerA and YpdA, and 2) to assess the substrate promiscuity of the FDR TrxB and its thioredoxin fold protein (TFP) partner, TrxA. These proteins, MerA, YpdA, TrxB and TrxA, may be involved in maintaining the redox balance in *S. aureus* through thiol disulfide interchange reactions.

These aims were be achieved by pursuing the following objectives:

1. Performing the cloning of the genes encoding the MerA, YpdA, TrxB and TrxA proteins.
2. Expression and purification of these four proteins
3. Executing an activity characterization of the MerA and YpdA proteins to determine their native substrates and oxidative stress-related function(s).
4. Assessing substrate promiscuity in the TrxB and TrxA proteins, as well as understanding the relevance thereof in *S. aureus* oxidative stress resistance.

In this manner, this study intends to provide a better understanding of *S. aureus*'s ability to resist oxidative stress, and to thereby lay the foundation for the development of novel anti-staphylococcal agents.

1.3.2 Outline of the thesis

The thesis consists of 5 chapters. The first chapter provides an overview of the study and states its aim and objectives. This is followed by a detailed review of our current knowledge of the *S. aureus* oxidative stress resistance mechanisms. Chapter 3 will discuss the purification and functional characterisation of the MerA and YpdA FDR proteins. Chapter 4 will detail the purification of the TrxB and TrxA protein system, as well as all experimental procedures indicating enzyme-substrate activity. The final chapter 5 will provide a summary of the findings in this study and detail any future work.

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Chapter 2

Oxidative stress resistance mechanisms of *Staphylococcus aureus*:

A review of the current knowledge

2.1 Introduction

Our current knowledge of *S. aureus* physiology indicates that this organism is capable of resisting oxidative stress via the orchestrated action of two distinct systems. As a first response, oxidative stress is dealt with directly by ROS-eliminating enzymes. These include superoxide dismutases to engage superoxide, whilst catalase and peroxiredoxins (the latter of which are thioredoxin-fold proteins or TFPs) degrade hydrogen peroxide.^{6, 7} However, while these enzymes can neutralise ROS, they cannot reverse any damage that the reactive molecules may have caused. Therefore, *S. aureus* employs a tight-knit thiol-disulfide interchange system to reduce any oxidised cysteine residues in proteins.⁴³

The aim of this chapter is to clearly outline the current knowledge regarding these systems, the various actors that are involved in them and their potential roles and interactions.

2.2 Mechanisms that directly eliminate ROS

2.2.1 Superoxide dismutase

Superoxide dismutases (SOD) are metalloproteins that catalyse the dismutation of $O_2^{\cdot-}$ to H_2O_2 and the release of O_2 in the process.^{4, 7, 24} These enzymes act as the first line of defence against ROS, converting $O_2^{\cdot-}$ to H_2O_2 , which in turn may be detoxified by catalase and peroxiredoxins.^{4, 7} The role of SODs in bacterial oxidative stress resistance is therefore crucial: the enzymes protect the cell against direct damage inflicted by $O_2^{\cdot-}$, but also indirectly prevent the formation of OH^{\cdot} (via the Haber-Weiss reaction of $O_2^{\cdot-}$ with Fe^{3+}).^{31, 40} *S. aureus* is known to possess two superoxide dismutases; SodA and SodM, both of which utilise manganese as their metal cofactor.⁸ Various studies have indicated that SodA is the primary superoxide dismutase of *S. aureus*, as mutants lacking *sodA* display greater superoxide sensitivity and growth defects than *sodM* mutants. However, both enzymes will function in concert to dismutate external sources of $O_2^{\cdot-}$.^{5, 6}

2.2.2 Catalase

H_2O_2 possesses the longest half-life of the primary ROS (milliseconds versus the nano- and pico-second half-lives of OH^\cdot and O_2^\cdot respectively).²⁴ This is due to the high stability of the molecule's oxygen-oxygen bond, which requires high activation energy in order to be broken, and the absence of any unpaired electrons. However, the O-O bond may be weakened by interaction with transition metals, most notably iron.^{5, 22}

The primary function of the catalase enzyme is the decomposition of H_2O_2 to H_2O and O_2 , thus protecting the cell against detrimental oxidation.^{22, 24} The enzyme is a homotetramer, containing one heme group per subunit. The heme prosthetic group found within catalase plays a very significant role in the enzyme's ability to break down H_2O_2 , as the O-O bond of H_2O_2 may be weakened by iron.^{5, 22} The reversible interaction of heme and H_2O_2 causes catalase to decompose H_2O_2 in two distinct steps (Figure 2.1)²²:

- 1.) The heme iron is oxidised by H_2O_2 , forming an enzyme-substrate intermediate often referred to as "compound I".
- 2.) A second H_2O_2 molecule is recruited to act as an electron donor to compound I, thereby regenerating the heme iron and allowing for the release of the final products, H_2O and O_2 .

As the second line of defence after SOD, catalase is responsible for fully neutralising the last of the ROS from the cell. Although H_2O_2 is the mildest and slowest of oxidants, its extended presence in the cell poses an integral threat until removed. Following the final elimination of all ROS, the bacterium may deploy the thiol-disulfide interchange system components to handle any oxidative damage sustained.

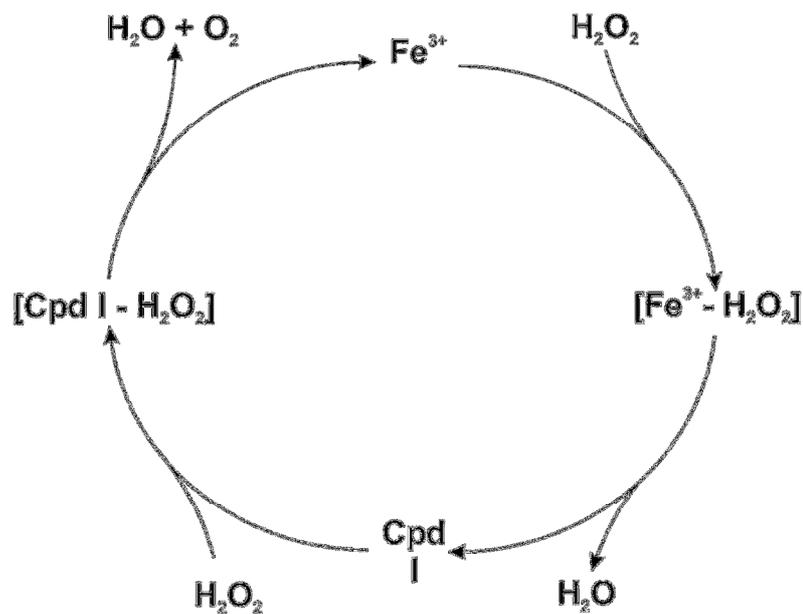


Figure 2.1: The neutralisation of H_2O_2 by catalase. The heme group of catalase is integral to the enzyme's mechanism of action, allowing for the formation of the "compound I" intermediate. Scheme modified from Nicholls et al.²²

2.2.3 Peroxiredoxins

Peroxiredoxins (Prxs) are thioredoxin-fold proteins (TFPs) that contain reactive cysteine residues that react with hydrogen peroxide to form sulfenic acid residues, and water.¹⁶ While these proteins therefore also directly eliminate ROS, they can only do so catalytically if their oxidised forms are reduced⁴³; this occurs by means of the thiol-disulfide interchange systems. They are therefore described in more detail together with other role players in this system (see section 2.3.2.3 below).

2.3 Redox balance maintenance through thiol-disulfide interchange reactions

The *S. aureus* thiol-disulfide interchange system makes use of low molecular weight (LMW) thiols, thioredoxin fold proteins (TFPs) and flavoprotein disulfide reductases (FDRs) to assist in the maintenance of a reduced intracellular environment. The role players of the thiol-disulfide interchange systems—like the Prxs mentioned above—must be constantly returned to a reduced state in order to function (Figure 2.2).⁴³

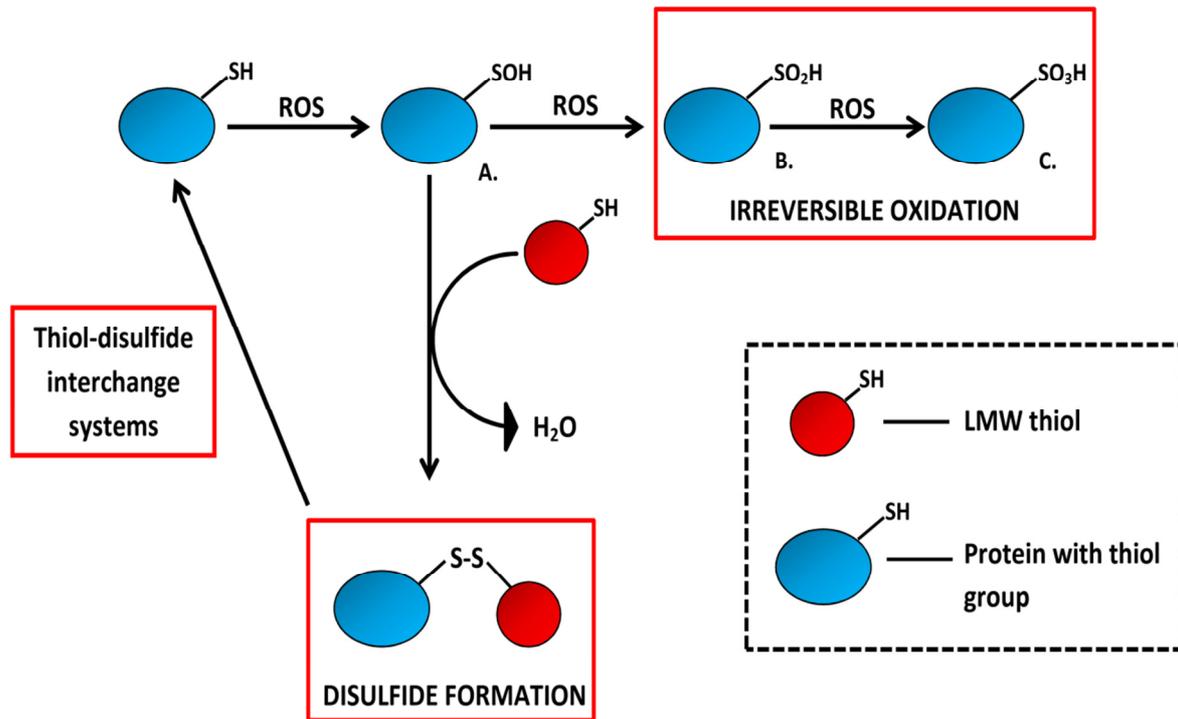


Figure 2.2: The effects of, and protection against, ROS on intracellular proteins. The initial reaction of a protein thiol groups with ROS leads to the formation of a sulfenic acid (A). Whilst sulfenic acid may be converted back to a normal thiol, prolonged exposure to ROS will lead to the formation of sulfinic and sulfonic groups (B and C). These groups are irreversible once formed, and the entire protein would need to be destroyed. In order to avoid this, LMW thiols are employed to form mixed disulfides with the thiol groups of proteins. The disulfide is protected from oxidation, and may be easily converted back to its original state via the thiol-disulfide interchange system.

When the bacterial cells are subjected to a blast of ROS by the human native immune response, they must be capable of both neutralising the reactive compounds and mitigating any damage they cause. For the former, the ROS-neutralising enzymes eliminate reactive intermediates. In order to avoid the crippling loss of essential enzymes and proteins, the cells rely on the working of the thiol-disulfide interchange system.

The thiol-disulfide interchange system serves the dual functions of protecting protein thiol groups from irreversible oxidation and restoring them to their native reduced state. LMW thiols may react with the sulfenic acid formed from initial ROS exposure, creating a mixed protein-LMW thiol disulfide. This process, known as S-thiolation, prevents the formation of irreversible sulfinic and sulfonic acids.²⁹ Once the cell has successfully eliminated the ROS,

the mixed disulfide must then be returned to its original reduced state through the action of FDRs and/or TFPs. Reversal of the S-thiolation is achieved in one of two possible ways^{29, 43} (Figure 2.3):

- The TFP may act first, transferring electrons (via the corresponding FDR) from NADPH to the disulfide bond between LMW thiol and protein, thus reducing it.
- Alternatively, the mixed disulfide bond may be reduced by an FDR directly, with NADPH again serving as the final electron donor in the process.

This system therefore insures that the intracellular proteins are both protected from irreparable damage, and returned to their original reduced state. The wasteful elimination of damaged proteins is thus kept to a minimum, and the bacterial cell may rapidly recover from the stresses endured.

However, our understanding of the thiol-disulfide interchange system of *S. aureus* remains incomplete. Whilst several players involved in the system have been assigned function, substrates and even interacting protein partners, many remain shrouded in the unknown. Structural analyses of the uncharacterised proteins have been taken together with an understanding of the general mechanisms whereby this system functions. This information has created a tentative roadmap whereby hypotheses surrounding uncharacterised proteins may be made. In the following section our current knowledge of these various role players will be reviewed.

2.3.1 The LMW thiols

S. aureus has been found to rely on two major LMW thiols for redox balance maintenance and xenobiotic detoxification: Coenzyme A (CoA) and bacillithiol (BSH).^{20, 21} This is contrast to most other organisms, which uses glutathione for these processes. However, *S. aureus* does not produce nor utilize this compound.^{20, 28}

CoA has been extensively researched for its roles in *S. aureus* redox homeostasis, while BSH was only recently discovered in *S. aureus*.²¹ The roles and known interactions of these LMW thiols in the organism's redox physiology are described below. Several other LMW thiols — such as cysteine, pantetheine and lipoamide — are also found in *S. aureus*,^{8, 35} but are not discussed in detail here since none of them have been implicated as being involved in maintaining its redox homeostasis to date. Cysteine in particular cannot practically be employed as a redox buffer due to its toxicity at high intracellular concentrations.^{8, 35} This toxicity stems from the phenomenon of rapid metal-catalysed auto-oxidation of cysteine in the cytoplasm, ultimately leading to the production of cystine and H₂O₂.^{3, 35} Lipoamide, on

the other hand, is usually physically bound to the protein that utilises it as a cofactor, and is therefore not considered as likely to interact with proteins other than its identified partners.^{18,}

27

2.3.1.1 CoA

The role of CoA in *S. aureus* was first brought to light in a study by Newton *et al.*²⁰ The study demonstrated that *S. aureus* does not synthesise the LMW thiol glutathione (GSH), and thus does not employ it for redox balancing mechanisms. Rather, the bacteria were found to produce millimolar quantities of coenzyme A (CoA), suggesting that the compound is the predominant thiol in *S. aureus*.^{20, 35}

Like GSH, CoA is resistant to metal-catalysed oxidation, and was also discovered to possess a dedicated FDR, CoA disulfide reductase, to maintain its reduced state.^{8, 35} However, unlike GSH, CoA cannot function as a reservoir from which Cys may be regenerated. Furthermore, the importance of CoA in *S. aureus* was demonstrated in a 2004 study by Leonardi *et al.*¹⁷ The biosynthesis of CoA in *S. aureus* was found to be resistant to feedback inhibition by CoA or any of its thioester derivatives, thus allowing the cell to maintain the millimolar concentrations of the cofactor required for redox homeostasis.¹⁷ Research into the natural antibiotic CJ-15,801 has provided more evidence to the importance of CoA in *S. aureus*. CJ-15,801, a structural homolog of pantothenic acid, was found to specifically inhibit the growth of *S. aureus* by targeting its CoA biosynthetic pathway.^{34, 36} The compound achieves this by irreversibly inhibiting the enzyme phosphopantothencysteine synthetase (PPCS), which catalyses the second step of CoA biosynthesis.³⁷ The evolution of a natural compound like CJ-15,801 is thus a testament to the importance of CoA to *S. aureus*'s survival; interruption of CoA biosynthesis is visibly detrimental to cell growth, making the pathway an obvious target.

Reviewing the information discussed, strong evidence is apparent for the importance of CoA in *S. aureus*. The maintenance of CoA at millimolar intracellular concentrations, the bacteria's lack of GSH and the negative effects observed during interruption of CoA biosynthesis; taken together, this information underscores the important role played by the thiol in *S. aureus*.

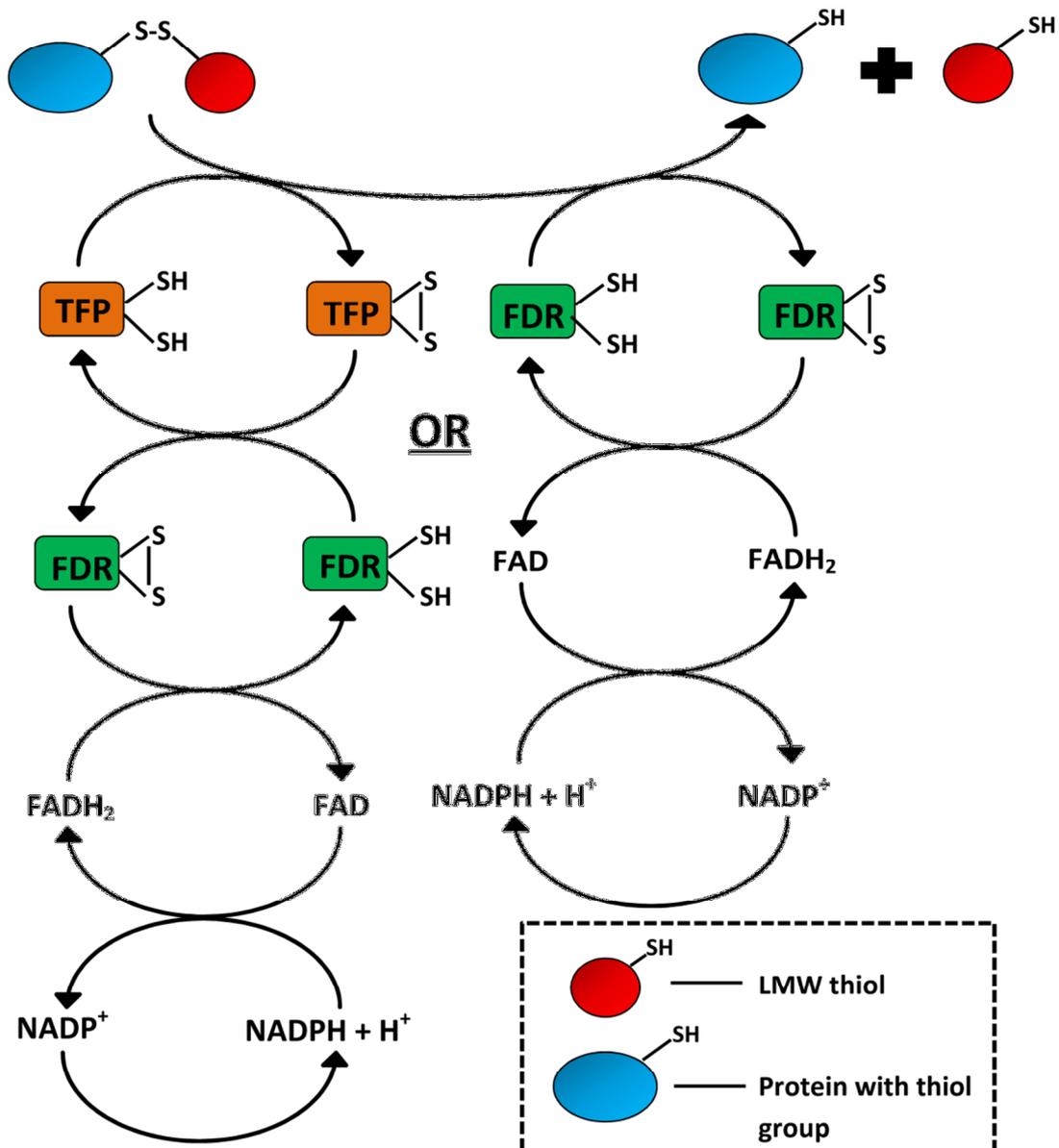


Figure 2.3: The general mechanisms of action for the thiol disulfide interchange proteins. The FDR protein may act either alone or in concert with its corresponding TFP partner to reduce the mixed LMW thiol protein thiol disulfide. When acting together, the TFP will reduce the mixed disulfide, becoming oxidised in the process. It will be returned to its reduced state through the action of its corresponding FDR. NADPH will donate electrons to the FAD subunit of the FDR, which in turn will reduce the oxidised active site Cys residues of the FDR. When acting without a TFP, the FDR acts directly upon the mixed disulfide. The resulting oxidised FDR will once again be returned to a reduced state via NADP and FAD.

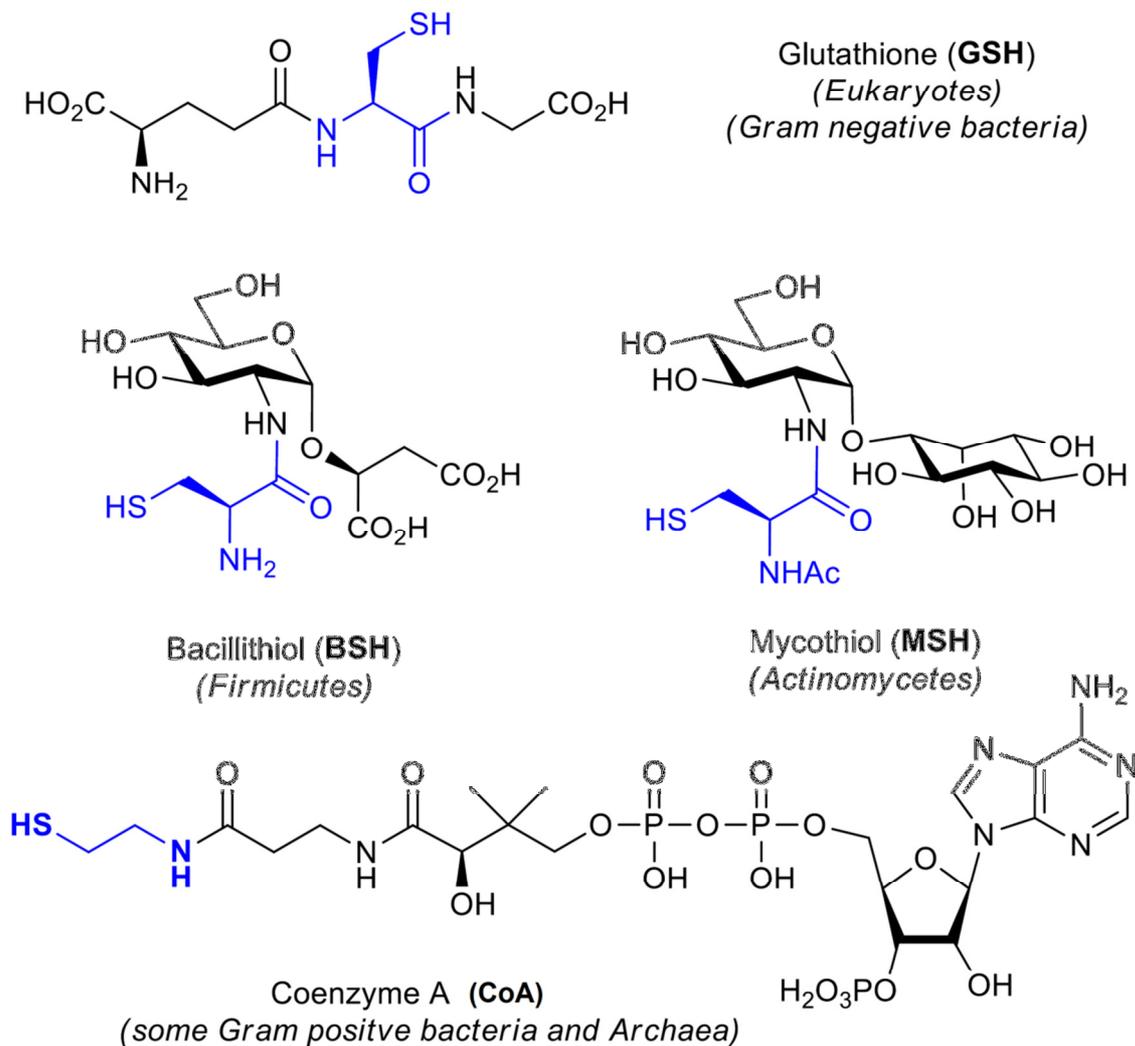


Figure 2.4: The major bacterial LMW thiols. Glutathione is the primary redox-active LMW thiol of eukaryotes and Gram-negative bacteria. Gram-positive bacteria however, contain several different LMW thiols among different species or classes. Mycothiol is utilised by the Actinobacteria, whilst bacillithiol may be commonly found within the phylum Firmicutes. CoA appears to act as the primary redox-active LMW thiol in several Gram-positive species of bacteria. Figure modified from Bui Khan, 2013.³

2.3.1.2 BSH

Bacillithiol was first reported by two independent studies as an unknown LMW thiol in *Bacillus* species.^{20, 21} Following the efforts of multiple collaborating researchers, it was structurally characterised and designated as bacillithiol, or BSH.²¹ Although BSH is currently considered to be the major LMW thiol of most *Bacillus* species, it has also been found to be present in most *Staphylococcus aureus* strains.³² Its structural analogy to mycothiol (the major LMW thiol of the Actinomycetes) has led to the hypothesis that BSH may play a significant role in the oxidative stress response of the bacteria that possess it.^{13, 32} Studies by Rajkarnikar *et al.* on *S. aureus* BSH-null mutants sought to explore the role of the LMW thiol in these bacteria. *S. aureus* BSH-null cells, similar to their *B. subtilis* counterparts, show increased sensitivity to thiol-oxidising reagents, reactive electrophiles, toxic metal ions & metalloids, and the antibiotics fosfomycin and rifamycin.³² Furthermore, when treated with cumene hydroperoxide and H₂O₂, BSH-null mutants proved to be very sensitive to oxidative stress. While these results are good indicators that BSH may be involved in maintaining *S. aureus* redox homeostasis, the exact role of the thiol in this process is uncertain.

A study by Pother *et al.* revealed certain strains of *S. aureus* that do not produce BSH, specifically the NCTC 8325 lineage and its descendents.²⁸ This is due to an 8 bp duplication in the *bshC* gene, which encodes the BSH-biosynthetic protein BshC. In accordance with the Rajkarnikar study, these natural BSH-null strains were found to be more sensitive to fosfomycin thiol-oxidising compounds such as hypochlorite.²⁸ Conversely, no difference was observed between the BSH-producing and BSH-lacking cells when exposed to diamide or H₂O₂.²⁸ However, the latter observation was performed under ideal cell culture conditions, and could lead to misleading interpretations. More appropriate results have been derived from observations that BSH-deficient *S. aureus* cells in the mouse macrophage model displayed greatly decreased viability.²⁸ These results together suggest that while *S. aureus* strains that are naturally unable to synthesise BSH are able to cope with oxidative stress, they do not do so nearly as well as those that do synthesise it. These results imply that BSH is not an essential LMW thiol for oxidative stress resistance in *S. aureus*. Rather, it may act as a back-up system in strains that can produce it, whilst also providing further virulence through fosfomycin resistance.

2.3.2 The thioredoxin fold proteins (TFPs)

The thioredoxin fold proteins are a class of protein superfamily linked by their use of a thioredoxin domain: a four-stranded β -sheet sandwiched by three α -helices.² A second

feature routinely shared among the TFP class is the presence of a CxxC active site associated with disulfide reduction. The latter, although not a ubiquitous feature, is commonly seen among the TFPs. TFPs are found in almost every living organism, and serve various functions, most often oxidative stress defence, assisting in protein folding, the formation/reduction of disulfide bonds and the elimination of xenobiotics.² The TFPs discussed in this section are primarily involved in redox balance and combatting oxidative stress in bacterial cells.

2.3.2.1 Thioredoxin (*S. aureus* TrxA)

Thioredoxin (Trx) is a multifunctional, LMW oxidoreductase possessing an active site containing two Cys residues. The most characteristic feature of all Trx proteins is that these residues are contained in a highly conserved CGPC motif.¹⁶ The motif's Cys pair is essential to Trx function; Trx reduces its disulfide-containing substrate (often a disulfide-containing protein), by thiol-disulfide interchange, which results in the oxidation of its Cys residues.^{14, 16} The reducing equivalents released in the process are thus donated to the substrate, and the Cys-pair of Trx forms a resultant disulfide bridge. Trx will subsequently be returned to its reduced state by thioredoxin reductase (TrxR) via a NADPH-dependent reaction (Figure 2.5). Hence, all Trx proteins rely on TrxR to return to a reduced state.^{1, 14}

For bacteria that do not make use of the glutathione/glutathione reductase system, the Trx-TrxR system is possibly the most crucial for maintaining intracellular redox regulation. The system is capable of reducing a wide variety of substrates simultaneously, while Trx alone has also been found to be involved in a number of diverse cellular functions, e.g. providing electrons to enzymes such as ribonucleotide reductase and methionine sulfoxide reductase.¹ The importance of Trx to the on-going survival of *S. aureus*, and indeed its success as a human pathogen, cannot be underestimated.

2.3.2.2 Peroxiredoxin

Peroxiredoxins (Prx) form part of a class of antioxidant enzymes known as the alkyl hydroperoxide reductases¹⁶. These proteins catalyse the direct reduction of H₂O₂ and organic hydroperoxides. The Prx system displays great similarity to the Trx system: most Prxs possess a similar active site CxxC motif that is required to carry out reduction of the substrate.^{25, 41} Additionally, the oxidised Prx relies on a NADPH-dependent reaction with its corresponding peroxiredoxin reductase (PrxR) to return to the reduced state. Peroxiredoxins may fall into one of three classes: typical two-cysteine, atypical two-cysteine and typical one-cysteine Prxs.⁴¹ Typical and atypical two-cysteine both contain two redox active Cys

residues, with the former presenting as a homodimer, whilst the latter is monomeric. Typical one-cysteine Prxs most likely require an exogenous thiol-containing electron donor with which to form a disulfide after reducing the substrate, although this has yet to be experimentally verified.^{16, 41}

The bacterial Prx, AhpC, is a typical two-cysteine Prx that functions with the PrxR, AhpF, in order to reduce peroxides. Although AhpC has been shown to demonstrate a lower catalytic efficiency towards H₂O₂ than that of bacterial catalase (10⁵ M⁻¹s⁻¹ vs. 10⁶ M⁻¹s⁻¹ respectively), it plays an important role in the detoxification of endogenously-produced peroxides.^{16, 41}

2.3.2.3 Glutaredoxin (and other LMW thiol-dependent redoxins)

Glutaredoxins (Grx) are glutathione-dependent oxidoreductases.¹⁶ Consequently, no examples of such proteins exist in *S. aureus*, as it contains no glutathione.²⁰ However, this family of proteins serve as a model for the interplay between a TFP and a LMW thiol, and as such will be discussed in detail here to provide background for the putative bacilliredoxins that might exist in *S. aureus*.

The Grx family of proteins share a number of characteristics with Trx: like Trx, they are proteins of a low molecular weight and are capable of acting upon a range of disulfide-containing substrates.¹⁶ However, the pathway of electron transfer in the Grx system is strikingly different from that employed by Trx (Figure 2.5).

The Grx pathway functions as follows¹⁶:

- Much like the Trx protein, Grx directly reduces a protein disulfide substrate, becoming oxidised in the process.
- The oxidised Grx is returned to the reduced state by GSH. This is in contrast to the oxidised Trx protein, which is reduced by a dedicated TrxR.
- The oxidised GSH (GSSG) is reduced by glutathione reductase (GR) in an NADPH-dependent reaction.

In addition to the different mechanism of action, Grx proteins are capable of functioning as either monothiol or dithiol enzymes, depending on the number of redox-active Cys residues present in the active site.¹⁶ The monothiol Grx protein catalyses the reduction of mixed disulfides, specifically those of glutionylated proteins. Dithiol Grx is capable of reducing a range of protein disulfides, and thus acts as a moonlighting redox-balance protein.¹⁶

For many species, the Grx/GSH/GR system is the primary means of combating oxidative stress and maintaining redox homeostasis. However, as stated above, *S. aureus* does not

contain GSH, and similarly lacks any Grx and GSSGR enzymes. Instead, the analogies drawn between BSH, GSH and the primary LMW thiol of the Actinobacteria, MSH, have led to the proposed existence of a “bacilliredoxin” (Brx) by Helmann.¹³ Such a Brx-dependent system has been suggested to act as a functional replacement for the Grx/GSH/GSSGR system in bacteria which lacks this LMW thiol. Furthermore, such a suggestion also implies the existence of a dedicated BSSB reductase of sorts. Thus, Brx proteins could potentially reduce mixed protein disulfides with BSH and a BSSB reductase (Figure 2.5). To date, no CoA-redoxins have been identified.

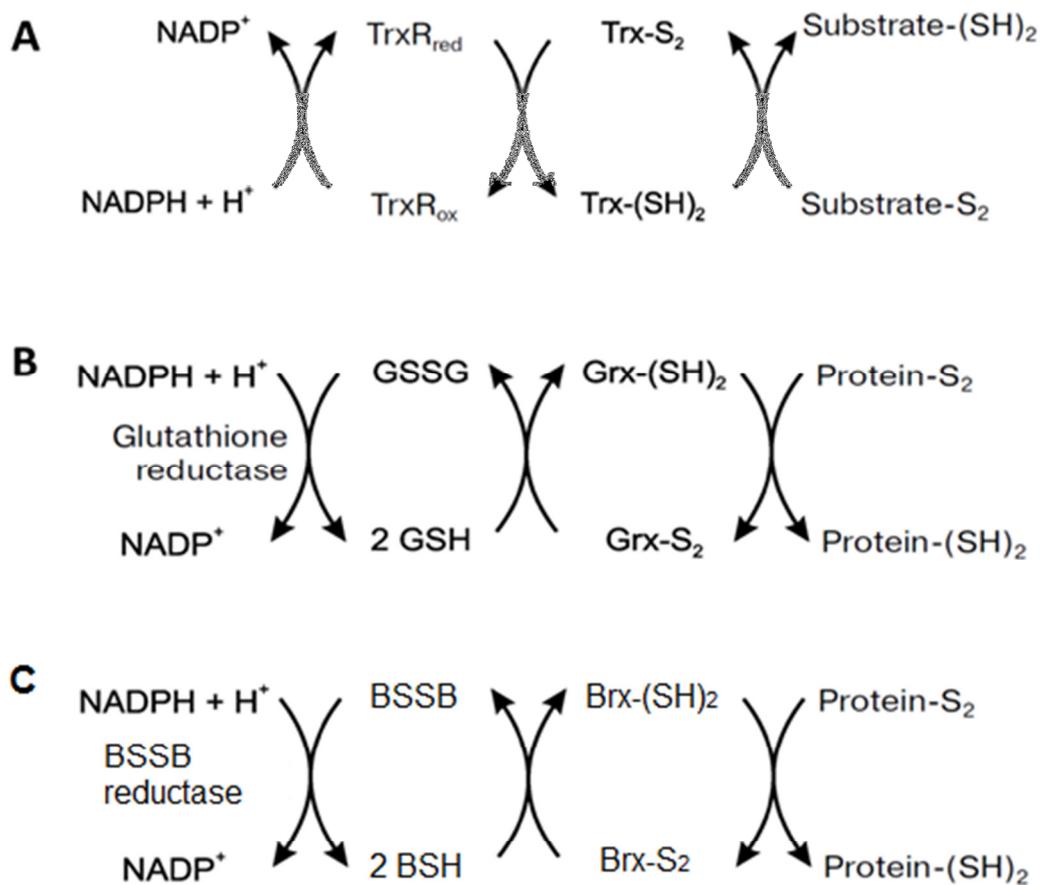


Figure 2.5: Grx vs. Trx mechanisms of action. A) Trx proteins reduce the mixed disulfide/protein disulfide substrate directly and are restored to the reduced state by the corresponding TrxR. **B)** Grx proteins also act directly upon the protein disulfide substrate. However the resulting oxidised Grx will not be reduced by a Grx disulfide. Instead, the oxidised Grx protein will be reduced by interaction with the LMW thiol GSH, which in turn is reduced by a dedicated glutathione reductase. **C)** A potential mechanism of action for the hypothesised “Brx/BSH/BSSB reductase system, based on the GSH model. Scheme adapted from Kalinina et al.¹⁶

2.3.2.4 Putative bacilliredoxins

Through phylogenomic profiling, a recent study by Gaballa *et al.* revealed a number of potential bacilliredoxins.¹⁰ The study utilised the EMBL STRING programmes to identify four conserved genes that occur with high frequency in the genomes of BSH-synthesising bacteria. The proteins of the genes identified are as follows: YpdA (a TrxR homolog, see section 2.3.4.3), YphP (a putative Trx-like protein), YqiW and YtxJ (a Grx-like protein).¹⁰

The *yqiW* and *yphP* genes are paralogues, thought to have both derived from the same ancestral gene and displaying 53% DNA sequence identity with one another.^{10, 13} YqiW and YphP both belong to the DUF1094 protein family, characterised by their small size (± 145 aa) and unknown function. DUF1094 proteins commonly occur in pairs that are widely conserved in BSH-producing Firmicutes. Although the YqiW and YphP proteins display several structural similarities to Trx proteins, both lack the critical active site CxxC motif. Instead, the proteins have a variant CxC sequence. YphP and YqiW have instead been suggested to potentially function as disulfide isomerases for BSH-related activities.¹³

In contrast, the YtxJ protein contains only a single conserved Cys residue, in a motif much like that seen in monothiol Grx. Previous studies have demonstrated that the expression YtxJ is induced by oxidative stress, although its role remains undiscovered.¹⁰

2.3.3 The flavoprotein disulfide reductases (FDRs)

The flavoprotein disulfide reductases are a group of proteins that catalyse oxidation and/or reduction reactions using a wide range of substrates. The FDRs are united by the presence of two dinucleotide binding domains in the form of Rossmann folds.²³ Whilst one domain always binds a FAD moiety, the other is capable of binding a pyridine nucleotide, i.e. either NADH or NADPH. The FDRs may be classified within one of three subgroups: the disulfide reductases (DSR), the alkylhydroperoxide reductases (AHR) or the peroxidase/oxidase subgroup (POR).²³

2.3.3.1 CoA disulfide reductase (CoADR)

CoA disulfide reductase (CoADR) is a dimeric flavoprotein primarily responsible for the direct FDR-only reduction of CoA disulfide in *S. aureus* (Figure 2.3).^{8, 35} At first glance, CoADR is structurally similar to proteins from the pyridine nucleotide disulfide oxidoreductase

(PNDOR) superfamily: it possesses two FAD-binding regions, one NADPH-binding region and a catalytic site, all of which are spatially organised similarly to proteins of the superfamily. Crucially lacking however, is a conserved pair of active site cysteine residues.^{8, 35} Instead, CoADR possesses only one Cys residue within the active site, whilst a second is found in the N-terminal $\beta\alpha\beta$ motif. Despite the presence of a second Cys, the active site Cys residue alone is involved in any catalytic redox activity.^{8, 35}

The single active site Cys residue of CoADR has drawn much interest. The Cys residue forms part of a SFxxC motif, most commonly seen in enzymes such as NADH oxidase or NADH peroxidase, which catalyse the formation and inactivation of H_2O_2 respectively.^{8, 35} Prior to discovery of the motif in CoADR, its presence was thought to indicate a role in the reduction of O_2 or a derivative thereof (e.g. H_2O_2). However, the appearance of the motif in a LMW thiol reductase suggests that it is indicative of a disulfide reductase.

Due to the crucial role of CoA as the primary LMW thiol active in maintaining *S. aureus* redox balance (see section 2.3.1.1), the equal importance of the CoADR enzyme must be emphasised as the dedicated FDR responsible for maintaining the reduced status of this LMW thiol. In spite of this central role, the inhibition of CoADR is not lethal to *S. aureus* as the *cdr* gene encoding the FDR is actually non-essential under normal growth conditions.^{8, 37} However, the inhibition of CoADR could still have catastrophic consequences for the cell during oxidative stress, although this has not specifically been investigated. Naturally, investigations into the development of CoADR inhibitors have therefore drawn some interest. The synthesis of the first CoADR inhibitors was carried out by van der Westhuyzen *et al.*, leading to the creation of four CoA analogues.³⁷ These analogues acted as competitive, irreversible inhibitors of CoADR through the modification of the single active site Cys residue. These inhibitors represent an important step in the work towards novel anti-staphylococcal agents targeting cellular redox balance.

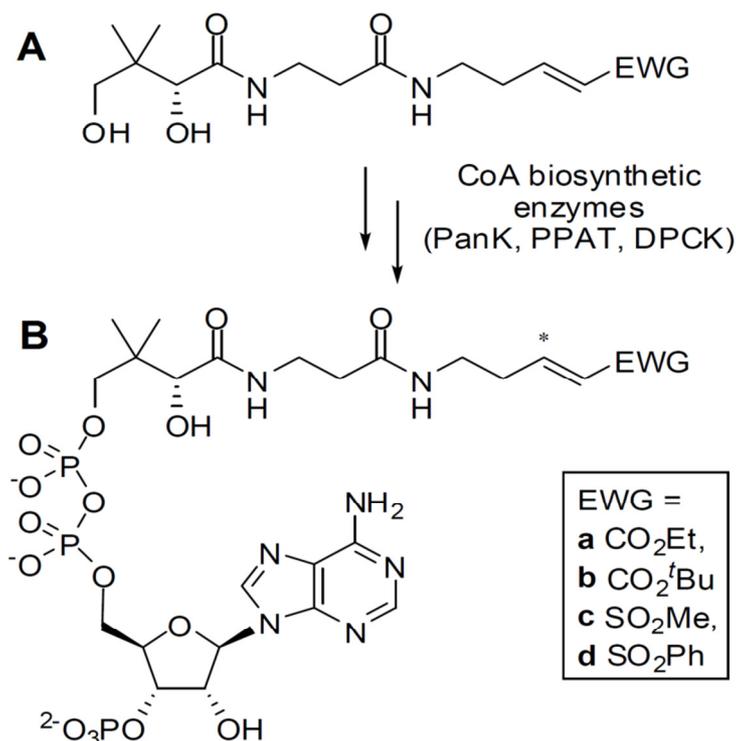


Figure 2.6: Michael-acceptor CoA and pantothenic acid analogs as inhibitors of CoADR. The CoA analogs designed by van der Westhuyzen et al. (**B**) are competitive inhibitors of CoADR. However, these compounds are too polar for cellular application, and thus their pantothenamide analogs (**A**) may be utilised. These compounds may cross the cell membrane and are converted to active CoA inhibitors by CoA biosynthetic enzymes. Scheme modified from van der Westhuyzen et al.³⁷

2.3.3.2 Thioredoxin reductase (*S. aureus* TrxB)

The thioredoxin reductase enzyme (TrxR) is a NADPH-dependent homodimeric oxidoreductase that reduces the active centre disulfide of oxidised Trx. TrxR contains a CxxC active site motif and one FAD molecule per subunit, both of which are critical to its reducing abilities. Together with NADPH, Trx and TrxR act as a powerful protein disulfide reductase system in most species that is capable of reducing a range of protein and LMW thiol disulfides in a non-specific manner.^{1, 14, 16}

The TrxR protein is essential to the survival of any living cell, with previous research efforts demonstrating that any attempts to create *trxR*-null cells promptly lead to cell death.³⁰ This is most likely due to the crucial role played by the thioredoxin system in DNA synthesis; Trx and TrxR channel redox equivalents to the class 1b ribonucleotide reductase, an enzyme centrally involved in DNA synthesis.^{1, 14} Furthermore, the “promiscuity” of all TrxR proteins is

well-documented. TrxR proteins have been demonstrated to interact with a wide range of substrates and other proteins, as well as fulfil several different roles in cellular function.^{1, 16}

The thioredoxin reductase of *S. aureus* (designated TrxB), together with its Trx partner (TrxA) drew the attention of this study due to their pivotal role in bacterial redox homeostasis. The *S. aureus* thioredoxin system makes an attractive subject of investigation: the above-mentioned substrate promiscuity of TrxR proteins has highlighted the importance of TrxB and TrxA during oxidative stress in *S. aureus*. Specifically the ability of *S. aureus* TrxB/TrxA to reduce CoAD or BSSB has not been explored by any previous study. Knowing whether these enzymes are capable of reducing these LMW thiol disulfides is especially important in the event that the FDRs that are normally responsible for their reduction are either inhibited or otherwise inactivated. If TrxB/TrxA could act as the LMW thiol reduction system of last resort for *S. aureus* under such circumstances, it would have important implications for the design of any novel antibiotics against this bacterium.

The importance of any TrxR to bacterial redox balance has naturally led to the investigation of potential inhibitors of its activity. Thus far, the most widely-studied inhibitor has been ebselen, a lipid-soluble seleno-organic compound that has been found to act as a competitive inhibitor of *E. coli* TrxR (Figure 2.7). The compound does not act as an inhibitor of TrxR, but instead competes with oxidised Trx for reduction by TrxR.⁴² Even more importantly; work by Holmgren *et al.* indicates that *S. aureus* is almost 100× more sensitive to ebselen than *E. coli*. This is suggested to be due to the finding that bacteria lacking the GSH disulfide-reducing systems, as *S. aureus* does, are more sensitive to ebselen.¹⁵

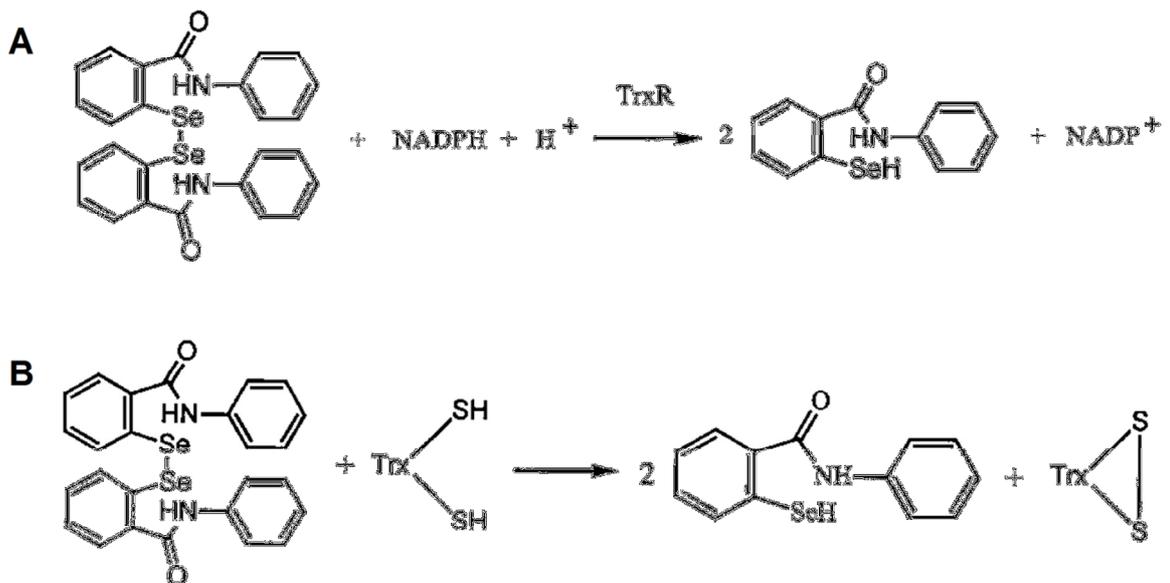


Figure 2.7: The inhibition of the thioredoxin system by ebselen. **A)** A dimer of ebselen can be reduced by the TrxR protein **B)** Ebselen does not act as a direct inhibitor of TrxR. Rather, it competes with the oxidised Trx protein to be reduced by TrxR. Scheme modified from Holmgren et al.¹⁵

2.3.3.3 Peroxiredoxin reductase (AhpF)

The bacterial peroxiredoxin reductase AhpF is a NADH-dependent peroxiredoxin reductase (PrxR) involved in the reduction of H₂O₂ and organic hydroperoxides.^{25, 26} It functions in tandem with the peroxiredoxin AhpC in a typical two-cysteine reaction, reducing the disulfide bridge formed between the redox active Cys residues of AhpC after interaction with peroxide. The Prx-PrxR system bears strong resemblance to that of Trx-TrxR, both in functional mechanisms and structure. Research on AhpF from *Salmonella typhimurium* suggests that the protein may have evolved from TrxR; a major portion of *S. typhimurium* AhpF displays up to 35% sequence identity to *E. coli* TrxR and possesses the same flavin-binding, pyridine nucleotide-binding and redox-active cystine motifs (CxxC).^{25, 26} Interestingly, whilst AhpC is ubiquitous across a number of species, AhpF is found strictly in eubacteria.

When the cell is subjected to an oxidative blast, AhpF and AhpC are involved in the direct elimination of ROS. Although not involved in the reduction of oxidised cellular proteins, the peroxide-reducing system does form part of the redox balancing mechanisms employed by *S. aureus* during oxidative stress.

2.3.4 FDRs of unknown function in oxidative stress resistance

Several FDRs have been identified within *S. aureus* that are projected to function in oxidative stress resistance: two DSR subgroup proteins (LipDH and MerA) and one AHR subgroup protein (YpdA). Whilst the function of the LipDH protein is well understood, MerA and YpdA's roles in *S. aureus* redox balance are unclear.

2.3.4.1 Dihydrolipoamide dehydrogenase (LipDH)

Dihydrolipoamide dehydrogenase (LipDH) is a member of the PNDOR family which forms part of the pyruvate dehydrogenase, α -ketoglutarate dehydrogenase and the branched-chain α -keto acid dehydrogenase complexes, specifically forming the E3 component.^{18, 27}

In each complex, the role of LipDH is the same: the enzyme catalyses the re-oxidation of the dihydrolipoamide moiety that is covalently bound to the acyl-transferase enzyme (E2) of the complex.^{18, 27} Because of this covalent linkage between LipDH and its substrate (or cofactor), it is unlikely that the enzyme will interact with free LMW thiols in the thiol-disulfide interchange system. Therefore, although LipDH is strictly also a thiol-disulfide reductase, it is not considered to be relevant in the maintenance of redox maintenance through thiol-disulfide interchange reactions, and was therefore not investigated in this study.

2.3.4.2 MerA

The *merA* gene of *S. aureus* was originally identified as a putative FDR through bioinformatic analysis of the bacterial genome. Additionally, a study by Voyich *et al.* revealed that the protein product of the *merA* gene (a MerA homolog) was heavily involved in the *S. aureus* oxidative stress response.³⁹ This was based on the significant up-regulation (more than 20-fold) of the gene following phagocytosis by neutrophils, and highlighted the MerA protein as a potentially important player in the *S. aureus* oxidative stress response.³⁹ However, the function of this protein and the role that it plays in oxidative stress resistance is not clear, as it shows significant homology towards known mercuric ion reductases (see Chapter 3). Such a function would not be relevant in the native environment encountered by *S. aureus*, and would not explain its role in eliminating ROS or ROS-inducing damage.

A comparative analysis of the amino acid sequences of the *S. aureus* MerA and known mercuric reductase enzymes reveals that the former lacks a penultimate Cys-Cys motif (Chapter 3). This characteristic is currently deemed to be essential for mercuric ion reductase activity, thus suggesting that MerA may fulfil other roles suited to a FDR.^{11, 19} A

basic BLAST analysis of *S. aureus* MerA further reveals significant sequence identity to FDRs such as dihydrolipoamide dehydrogenase and glutathione reductase, which also belong to the DSR subgroup.⁹ The MerA homolog is thus similar in sequence to FDRs that do not reduce a corresponding TFP partner, but which rather reduce an oxidized substrate (usually a LMW disulfide) directly. It was therefore concluded that MerA may act as a BSSB reductase or as an alternate CoAD reductase, the latter activity explaining the apparent non-essentiality of the CoADR enzyme.

From the information discussed above, it is clear that the role of MerA in the oxidative stress response of *S. aureus* is both very important, yet still very uncertain. The characterisation of this protein may shed much light on the defence mechanisms employed by *S. aureus* when it experiences oxidative stress.

2.3.4.3 YpdA

The YpdA protein was first identified by Gaballa *et al.* as a FDR with a potential role in BSH-dependent processes in *B. subtilis*.¹⁰ Identified by the EMBL STRING programmes, the *ypdA* gene was one of four found to occur with high frequency in the genomes of BSH-synthesising bacteria. Bioinformatic studies of the *S. aureus* genome later revealed a YpdA homolog, displaying 62% identity to that of the *B. subtilis* counterpart.¹³

Furthermore, YpdA shares strong identity with known thioredoxin reductases, and falls within the same AHR subgroup of FDRs as this enzyme.^{10, 13} Due to the structural similarities displayed by YpdA to the above-mentioned enzymes, it is most likely that the FDR requires a corresponding TFP (most likely a thioredoxin) in order to carry out its functions. This led to the conclusion that YpdA could be responsible for the reduction of oxidized bacilliredoxin proteins. However, it has also been proposed as a candidate for the as yet unidentified BSSB reductase. Analysis of YpdA's sequence (by comparison to other FDRs of known function) highlights several potential problems with such an analysis (see Chapter 3): first, the protein lacks the CxxC active site motif required to perform such functions, strongly indicating that it should be inactive as a disulfide reductase (if it follows the standard mechanism of these enzymes). Second, the protein was found to possess a sequence insert within its NADPH-binding domain, which may potentially affect its ability to bind the pyridine nucleotide. Finally, despite many studies echoing the theories postulated by Gaballa *et al.*, no BSH-associated activity has been experimentally verified for any YpdA, including the *S. aureus* homolog.

2.3.4.4 Summary of the thiol-disulfide interchange mechanisms in *S. aureus*

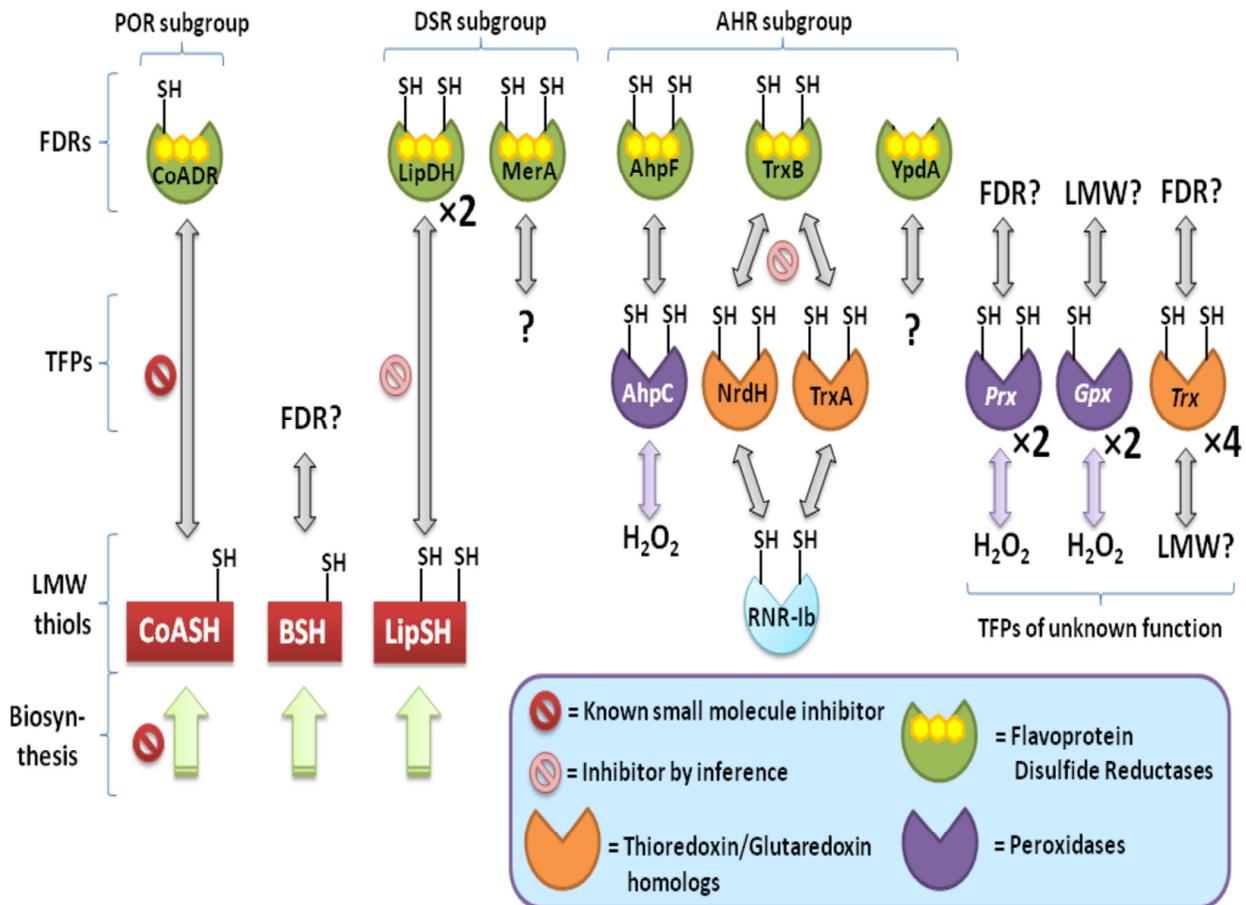


Figure 2.8: The currently understood thiol redox balancing mechanisms of *S. aureus*. FDRs, TFPs and LMW thiols form the three principal components of the system. Each reduces the oxidised version of the other in order to maintain intracellular redox balance. As may be seen above many gaps in the current knowledge of the system prevent the picture from being completed.

2.4 Unresolved questions in *S. aureus* redox homeostasis

As can be summarised from the evidence discussed, many gaps remain to be filled in our understanding of the redox balancing mechanisms of *S. aureus*. Of these, determining the native functions of the MerA and YpdA proteins represent the greatest challenge, given that their roles in the thiol-disulfide interchange system can thus far only be guessed. The main question for both is the same: what role(s) do these two enzymes play in the oxidative stress resistance mechanisms of *S. aureus*? In the case of MerA, is it a functional mercuric ion

reductase, despite anomalies in its amino acid sequence? If so, what use could a mercuric ion reductase possibly be during oxidative stress? In the case of YpdA, could it be the elusive bacillithiol reductase? Does it require a corresponding TFP to function? And if not bacillithiol, then which substrate does YpdA act upon?

TrxB also raises questions, although of a different nature: Is its substrate spectrum wide enough that it (together with TrxA) could act as a back-up system for CoADR by acting upon CoAD? Or could it act on BSSB as a substrate in a similar manner? What other proteins and/or LMW thiol disulfides is it capable of reducing?

As outlined in Chapter 1, answering these questions forms the basis of this study. Fruitful research efforts in this regard will allow for a greater understanding of the varied mechanisms employed by *S. aureus* during oxidative stress. Most importantly, the findings drawn from this study may help lay the foundations for the development of novel anti-staphylococcal agents, of which the world is sorely in need.

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Chapter 3

Cloning, expression, purification and characterisation of the MerA and YpdA homolog proteins

3.1 Introduction

At the crux of this study lie the questions surrounding the function and activity of MerA and YpdA homologs. As functionally uncharacterised FDRs, they represent the greatest unknown in the model of *S. aureus* oxidative stress resistance.

The MerA homolog is of particular interest based on the significant up-regulation of the *merA* gene following phagocytosis by neutrophils (and therefore presumably after exposure to oxidative stress), which suggests that it plays a vital role under these conditions.²² However, since MerA shows greatest homology to known mercuric ion reductases, such a conclusion would be puzzling since such an activity should have minimal importance in the organism's defense mechanisms against oxidative stress. MerA is therefore likely to play a vital role in oxidative stress resistance that may be unrelated to functions associated with its homology to mercuric ion reductases.

The implied link between YpdA and BSH is another important point of interest. Although suggested to be a BSSB reductase or bacilliredoxin, the YpdA protein has yet to be experimentally verified as such.¹² Providing conclusive evidence to such claims would shed much light on the role of BSH in *S. aureus*.

This chapter will discuss all experimental attempts to assign function to the MerA and YpdA proteins of *S. aureus*, specifically with regards to their potential roles in combatting oxidative stress.

3.2 Results

3.2.1 Sequence analysis of the MerA homolog by comparison to known mercuric ion reductases

The *S. aureus* MerA homolog is labelled as such based on its sequence similarity with flavoproteins that are known to reduce Hg^{2+} to Hg^0 , i.e. mercuric ion reductases. These proteins have been studied intently for more than 30 years, and are known to possess several distinctive sequence characteristics. These include 1) a redox active Cys pair

located in the active site, 2) a long N-terminal extension possessing a Cys pair and 3) a shorter C-terminal extension possessing a Cys pair. Additionally, like all FDR's the proteins also have one NADPH-binding domain and one FAD-binding domain per subunit³ (Figure 3.1).

The amino acid sequence of MerA was aligned with the sequences of two known mercuric ion reductases from different bacterial species to establish the level of its similarity with these proteins (Figure 3.2). This alignment reveals several important differences between MerA and other known mercuric ion reductases. The most obvious is the drastic difference in size between MerA and the other sequences. A large portion of the N-terminal sequence of the known mercuric reductases is missing in MerA, including an N-terminal cysteine pair. Most critical however, is the lack of the penultimate cysteine pair near the C-terminal.

Extensive research on the N-terminal domain of mercuric reductase (NmerA)^{3, 4, 20} indicated that whilst the N-terminal Cys pair is dispensable under normal, non-stressed conditions, mutations of the C-terminal Cys pair drastically reduces enzymatic activity.^{3, 19} More specifically it was found that a double Cys→Ala mutant displays no catalytic activity whatsoever, while mutation of only one of the Cys residues cause significant decreases in k_{cat} and k_{cat}/K_m values.^{3, 19}

These results, taken together with X-ray structure work by Brown *et al.*, suggest that the C-terminal Cys pair is critically involved with the acquisition of Hg²⁺ ions in the cell.^{3, 4} The loss or mutation of these residues can thus severely affect enzyme efficiency. The conclusions drawn from these studies suggest that a mercuric ion reductase lacking the penultimate Cys pair, as MerA does, would be incapable of reducing Hg²⁺ ions.

From the sequence analysis of MerA, it is plain to see that the protein lacks several key sequence motifs and features, most notably the C-terminal Cys pair. Based on this, it is not entirely clear whether the protein is capable of normal mercuric ion reductase activity. In spite of this, MerA does contain three other core features of mercuric ion reductases: a core active site Cys pair, and both NADPH- and FAD-binding domains. The presence of the above features may be sufficient to allow MerA to function, albeit at an impaired rate.

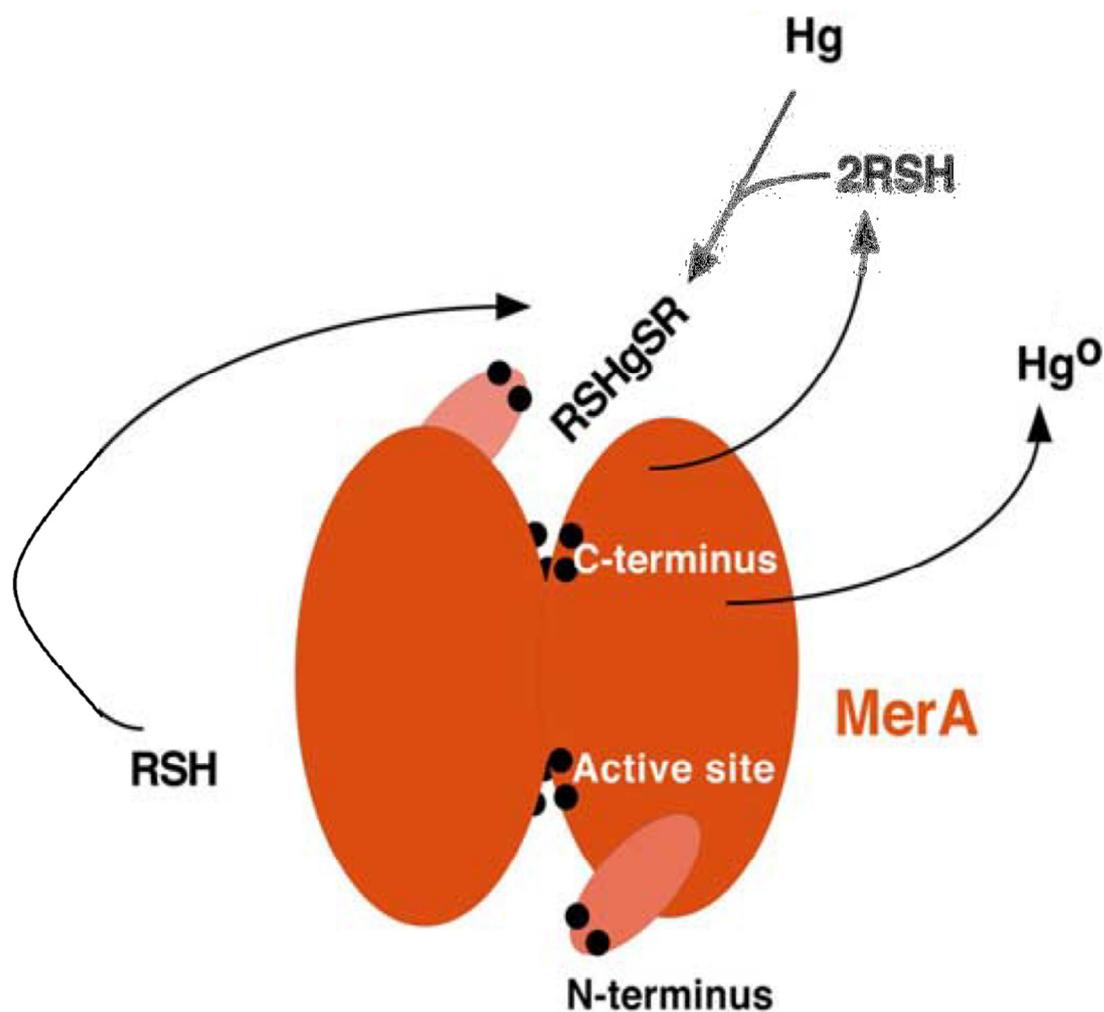


Figure 3.1: Schematic representation of the 3D structure of known mercuric ion reductases. The black dots indicate cysteine residues. Mercuric ion reductases possess Cys pairs in the N- and C-terminals, as well the active site. Scheme adopted from Barkay et al.³

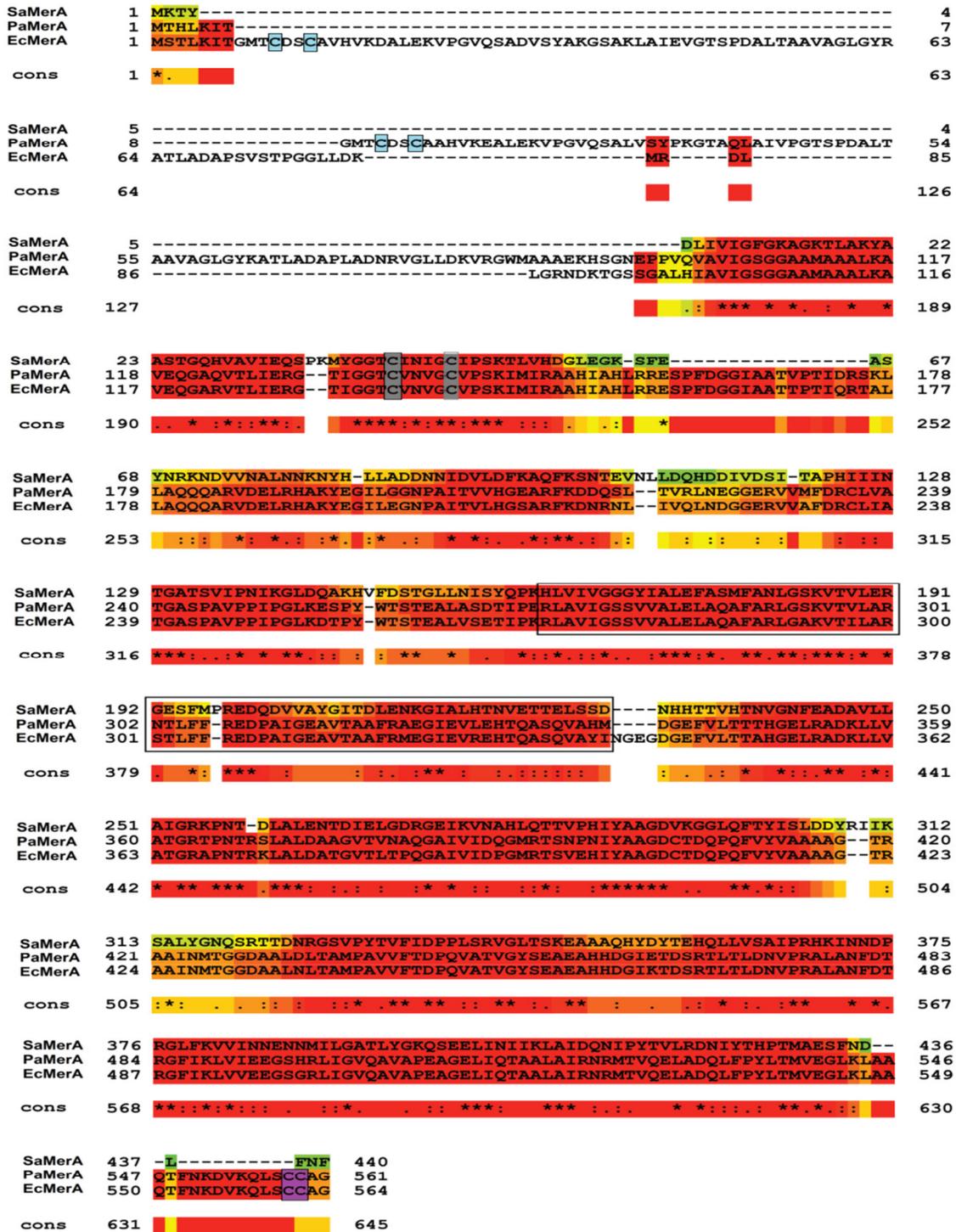


Figure 3.2: T-coffee amino acid sequence alignment comparing MerA to known mercuric ion reductases from *P. aeruginosa* and *E. coli*. The N-terminal and C-terminal Cys pairs are marked in blue and purple respectively. The active site Cys pairs are marked in grey, and the NADPH-binding domain is outlined in black. The FAD-binding domain stretches between residues 100-407.

3.2.2 Sequence analysis of YpdA by comparison to known thioredoxin reductases

YpdA has been suggested as the candidate protein for both BSSB reductase and bacilliredoxin reductase activity.¹² Preliminary sequence analysis indicated that YpdA shows the highest similarity to FDRs of the Ahr subgroup¹², and its amino acid sequence was therefore aligned with that of a well-characterized member of this group, thioredoxin reductase (TrxB), as well as a unusual TrxR protein identified in *T. acidophilum* (*TaTrxR*) (Figure 3.3). The latter was chosen as a protein similar to YpdA, in the sense that it is a putative TrxR lacking the known catalytic motifs associated with such a protein.

Aligning the amino acid sequences of YpdA and TrxB illustrates multiple similarities, but also differences between the proteins. The most obvious and important difference is the lack of a redox-active Cys pair in YpdA; instead of the typical CxxC motif displayed by both TrxB and *TaTrxR*, it has a FKEA sequence, which seems to bear no functional relevance. The YpdA protein does possess two cysteine residues elsewhere in its sequence, although they are not located in close proximity with one being found close to the N-terminus, and the other close to the C-terminus. As discussed in Chapter 2, the CxxC motif is considered essential to the catalytic activity of a TrxR protein, and the anomaly in YpdA should therefore render it inactive as a disulfide reductase. This suggests that it either has another unrelated activity, or it does not follow the standard mechanism for the reduction of protein and/or LMW disulfides.

Additionally, both YpdA and the *TaTrxR* display variation in their NADPH binding domains compared to that of TrxB. Comparing the TrxB NADPH-binding domain located between residues 146 and 219 to those of the other proteins, clearly confirms the previous finding that *TaTrxR* has not maintained the standard NADPH-binding motif: instead of the usual VxxxHRRDxxRA sequence, *TaTrxR* has VxxxEYMPxxMC.¹³ This drastically influences the electrostatics of its binding pocket, and consequently it was found to bind neither NADH nor NADPH. For the corresponding sequence, YpdA has a VxxxYRGGxxSP motif that retains at least one Arg residue, while also only introducing small glycine residues. This suggests that unlike *TaTrxR*, YpdA may still bind NADH and/or NADPH, which could lead to the reduction of its FAD cofactor.

3.2.3 Gene amplification and plasmid construction

The *merA* gene (SAOUHSC_00581) was amplified from genomic *Staphylococcus aureus* DNA strain RN4220. An N-terminal NcoI site and a C-terminal XhoI site were introduced to the gene during amplification, thus allowing *merA* to be cloned into the pET28a(+)

expression vector. The restriction sites introduced were chosen such that MerA would be expressed with a C-terminal His-tag. The successful formation of a pET28a(+)-merA recombinant plasmid was confirmed via digestion screening.

The ypdA gene (SAOUHSC_01499) was amplified and inserted into the pET28a(+) expression vector in a similar manner.

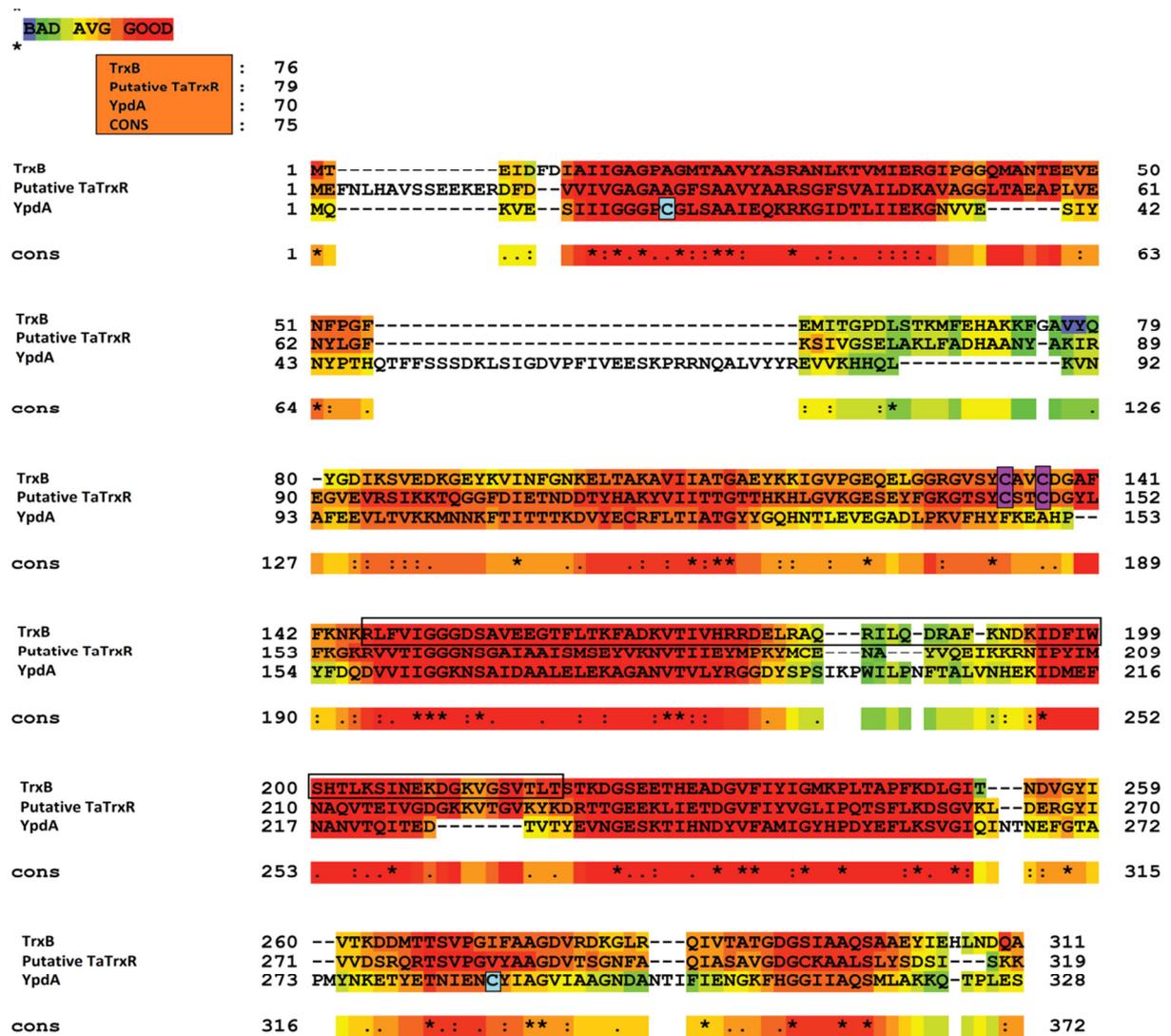


Figure 3.3: T-coffee amino acid sequence alignment comparing YpdA to TrxB and a putative TrxR from *T. acidophilum* (TaTrxR). The active site Cys pair of TrxB and TaTrxR are marked in purple, whilst the unpaired Cys residues of YpdA are marked in blue. The NADPH-binding domain of TrxB is outlined in black. The FAD-binding domain stretches from residues 7-283.

3.2.4 Heterologous expression trials

Expression trials were carried out in order to elucidate the optimum expression parameters for soluble expression of MerA. The trials were conducted using *E. coli* BL21* (DE3) cells, and expression was allowed to continue overnight. Optimum results were obtained at 22°C, with a total IPTG concentration of 0.5 mM, as can be seen from the SDS-PAGE analysis shown in Figure 3.4.

Expression trials for YpdA were carried out as described above for MerA. Optimum expression was obtained at 37°C, with a total IPTG concentration of 0.5 mM, as can be seen from the SDS-PAGE analysis shown in Figure 3.5.

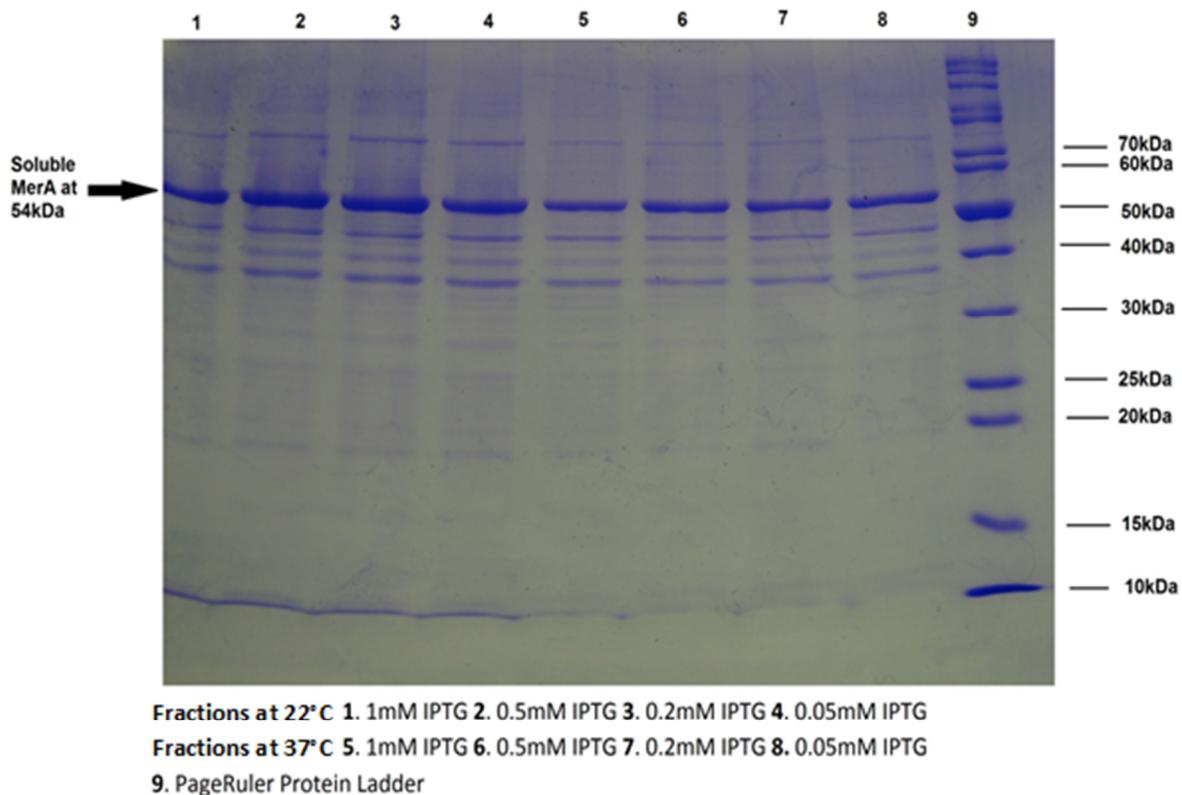


Figure 3.4: SDS-PAGE of soluble expressed MerA protein. The crude cell extracts from different expression conditions were separated by SDS-PAGE on a 12% gel. The MerA protein is tentatively identified based on it having the expected size (as judged by comparison to the molecular weight markers) and is indicated by the arrow..

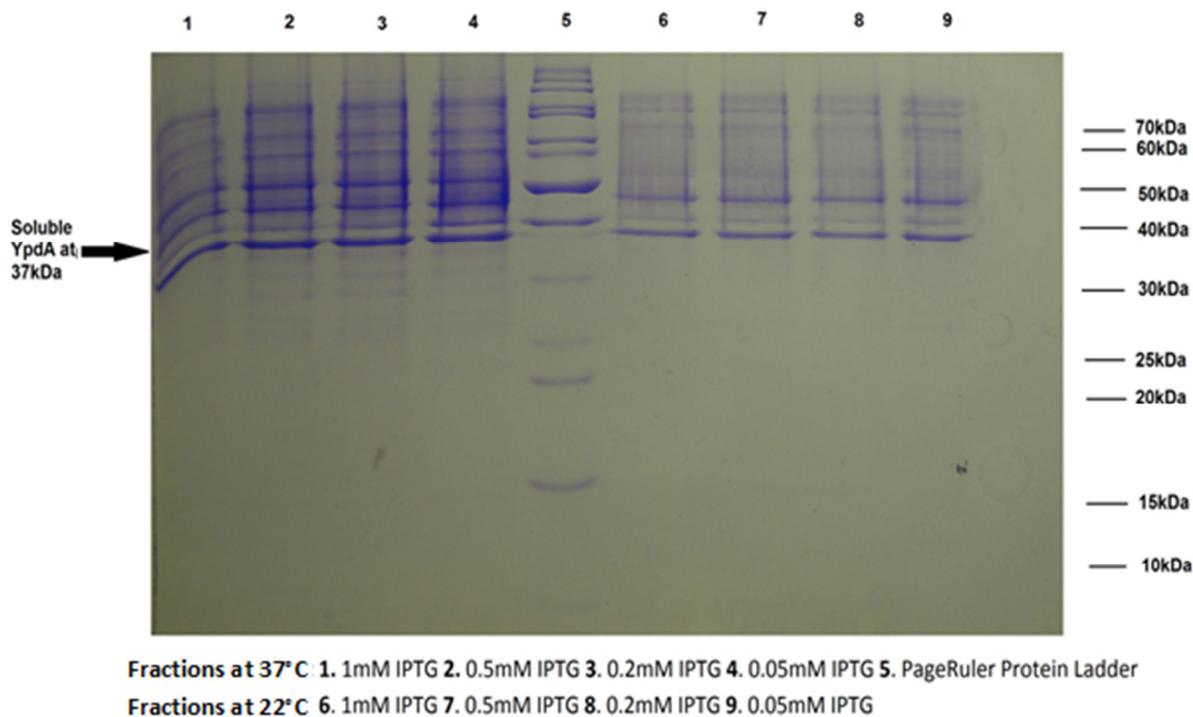


Figure 3.5: SDS-PAGE of soluble expressed YpdA protein. The crude cell extracts from different expression conditions were separated by SDS-PAGE on a 12% gel. The YpdA protein is tentatively identified based on it having the expected size (as judged by comparison to the molecular weight markers) and is indicated by the arrow..

3.2.5 Protein purification

The ideal expression temperatures determined above were used in a large-scale expression of MerA. Purification of the protein from bacterial lysate was performed by immobilized metal affinity chromatography (IMAC) on an ÄKTAprime system using a 1 mL HiTrap Chelating column that was loaded with Ni²⁺ prior to purification (Figure 3.6)

The crude lysate containing the His-tagged protein was loaded onto the column and all non-specific bound proteins were washed off. MerA was eluted by increasing the imidazole concentration to 500 mM in the elution buffer (20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 7.9). The imidazole was removed by buffer exchange using a 5 mL HiTrap desalting column and a low salt buffer (25 mM Tris-HCl, 5 mM MgCl₂, pH 8.0). Protein elution profiles were monitored via UV detection at 254 nm. The protein concentration of MerA was determined by a Bradford assay to be 5.65 mg/ml. Additionally, the purified protein was bright yellow, indicative of it containing the FAD-cofactor.

The large-scale expression of YpdA was carried out using the parameters determined through expression trials. Purification of YpdA was conducted in the same manner as that of MerA (described above), both through IMAC and gel filtration. The protein concentration of YpdA was determined by a Bradford assay to be 3.58mg/ml (Figure 3.6)

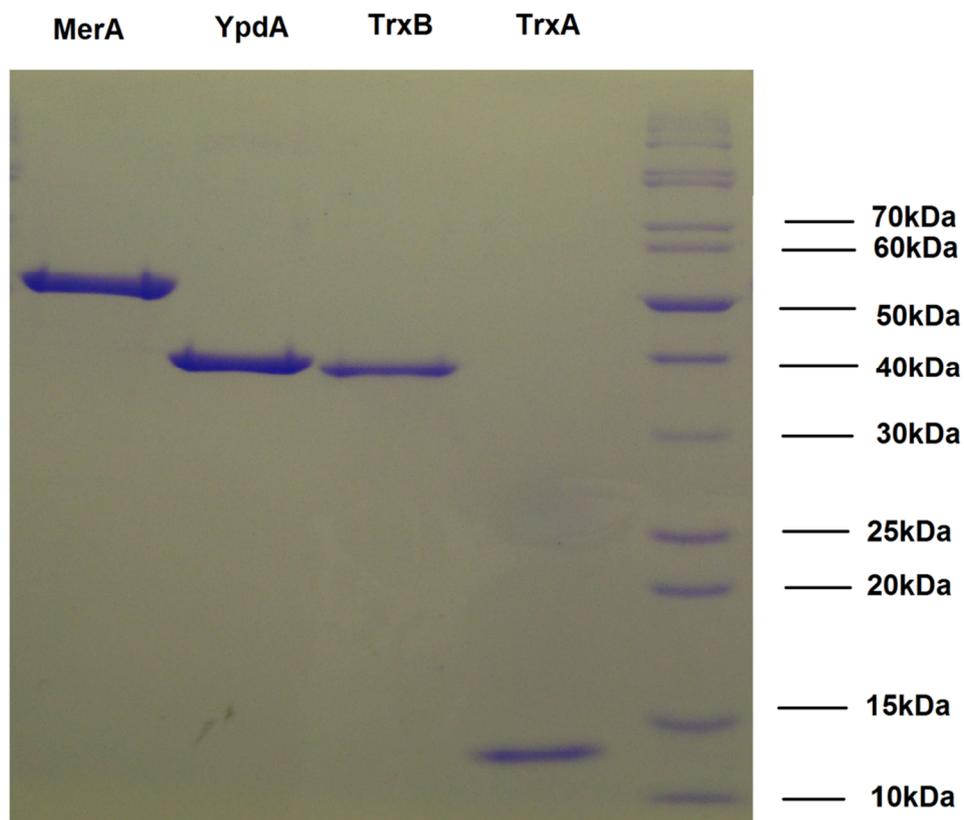


Figure 3.6: SDS-PAGE analysis of soluble MerA and YpdA protein after IMAC and gel filtration purification. The gel also shows the TrxB and TrxA proteins purified as part of the studies described in chapter 4.

3.2.6 Determining the redox cofactor requirements of MerA and YpdA

UV-Vis analysis of the purified MerA and YpdA proteins showed that both gave the characteristic spectrum of a flavin cofactor. To determine whether this cofactor can be reduced by the binding of NADH and/or NADPH, anaerobic titrations were conducted and compared to that of a control, the *S. aureus* TrxB enzyme. This was done in a manner similar to that previously employed to study the cofactor requirement of the *Ta*TrxR enzyme.¹³

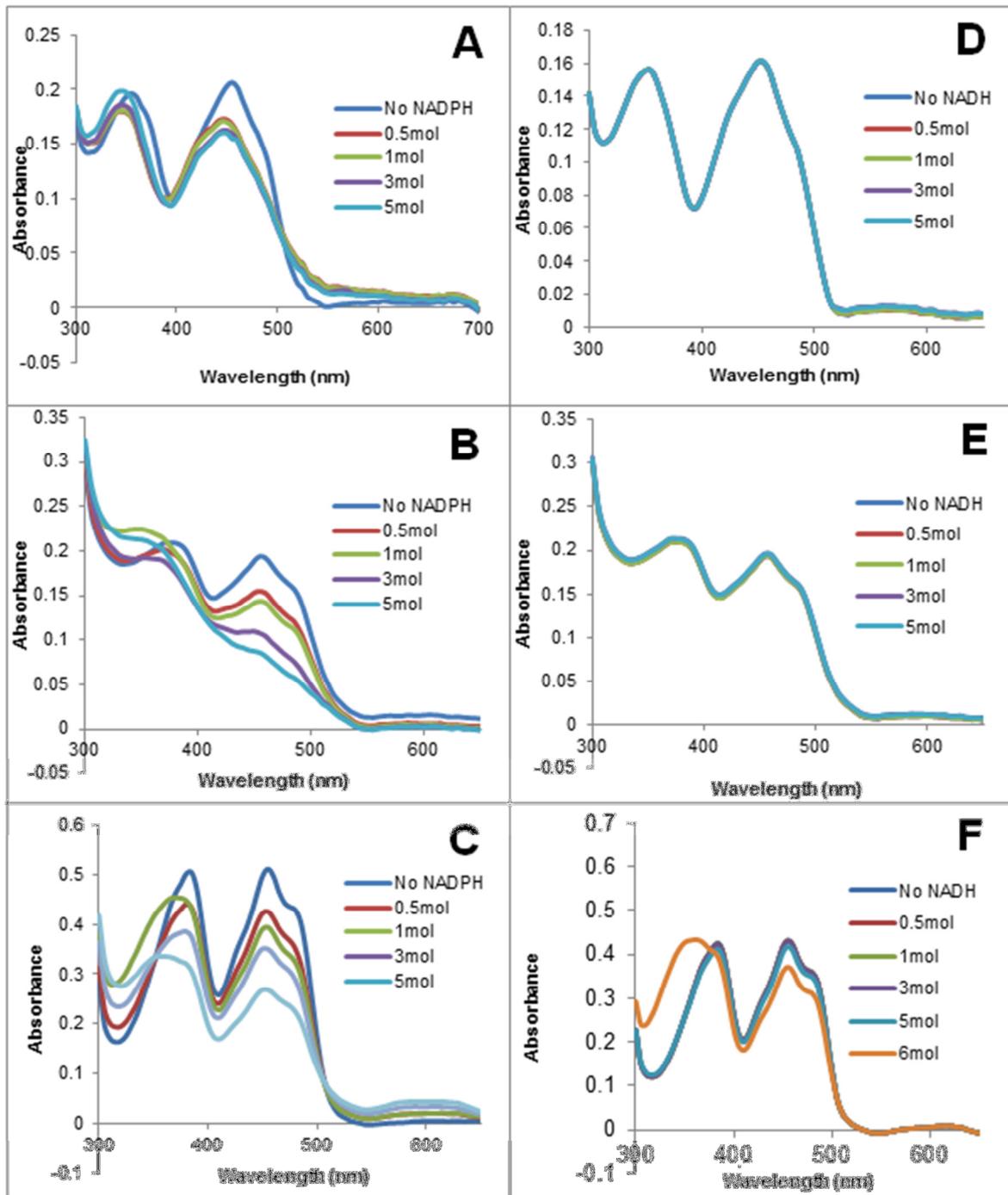


Figure 3.7: Anaerobic titrations of MerA and YpdA with NADPH and NADH. **A)** MerA titrated with NADPH. **B)** YpdA titrated with NADPH **C)** TrxB titrated with NADPH. **D)** MerA titrated with NADH. **E)** YpdA titrated with NADH **F)** TrxB titrated with NADH. The proteins were titrated via the sequential addition of 0.5 mol NADH/NADPH per 1 mol flavin moiety, until an excess of 5 mol NADH/NADPH per 1 mol flavin was reached.

The results show that the flavin moiety of MerA was readily reduced in the presence of NADPH, with the absorbance at 450 nm decreasing immediately at the addition of 0.5 mol of NADPH per 1 mol flavin moiety (Figure 3.7A), which is similar to the response shown by TrxB (Figure 3.7C). However, unlike TrxB MerA's flavin does not seem to be fully reduced even after treatment with 5 eq. NADPH. MerA displayed no reaction towards NADH, even at molar concentrations in excess of 10-fold (Figure 3.7D), while TrxB did show some reduction with NADH, although only when an excess (6 mol eq) were added (Figure 3.7F). This confirms that MerA, like TrxB, prefers NADPH as the reductant of its flavin cofactor.

For YpdA, anaerobic titrations showed that its flavin cofactor was rapidly reduced in the presence of NADPH, with the absorbance at 450 nm decreasing noticeably with as little as 0.5-fold molar excess of NADPH (Figure 3.7B). At 5 eq. NADPH, it was fully reduced. In contrast, YpdA displayed no reaction towards NADH, even at molar concentrations in excess of 10-fold. This demonstrates that YpdA has the ability to bind redox cofactors (with a similar preference as TrxB) in spite of the modifications in its NADPH-binding motif highlighted above.

3.2.7. Determination of standard assay conditions

With the knowledge of the respective enzymes' preferred redox cofactors in hand, we could next evaluate their activity towards various substrates. Since neither enzyme has been characterized before, we decided to use the same assay conditions used for the only characterised non-TFP-requiring FDR in the *S. aureus* thiol-disulfide redox system, namely CoADR. However, this enzyme has been previously been assayed using two different assay conditions.¹⁷ These conditions are compared in Table 3.1 below.

To determine which of these conditions gave the best result in our hands, the rate of reduction of CoAD by CoADR was monitored spectrophotometrically in 96-well plates by following the decrease in NADPH absorbance at 340nm. The assay was conducted using both conditions, and the initial reaction rates determined for each. This gave the activity profiles shown in Figures 3.8A and 3.8B.

Both sets of assay data were subsequently fitted to the Michaelis-Menten equation:

$$v_0 = \frac{V_{\max}[S]}{K_M + [S]}$$

Where v_0 represents initial velocity of the reaction, V_{\max} is the maximal reaction velocity and K_M is the Michaelis constant. In the Michaelis-Menten equation, V_{\max} occurs at high substrate concentrations, whilst K_M is equal to the substrate concentration when the reaction velocity is half-maximal.

Catalytic turnover, or k_{cat} , is defined as the maximum number of moles of substrate that may be converted to product, per mole of enzyme, in units of time. Catalytic turnover is calculated as follows:

$$k_{\text{cat}} = \frac{V_{\max}}{[E]_{\text{total}}}$$

Henceforth, all kinetic parameters determined for the various reactions in this study were calculated as described above.

Table 3.1: Comparison of published CoADR assay conditions

Components	Potassium Phosphate Conditions ¹⁷	HEPES Conditions ²³
Buffer base	50 mM K ₂ HPO ₄ (pH 7.0)	40 mM HEPES (pH 7.5)
EDTA	0.5 mM	1.0 mM
NADPH	32 μM	150 μM
Enzyme	20 nM	20 nM
Initiator (substrate)	600μM CoAD	600μM CoAD
Temperature	25°C	37°C
Duration	2mins	10mins
Wavelength	340nm	340nm

The results are summarized in Table 3.2. Although both assays generated very similar kinetic data, the potassium phosphate conditions were ultimately selected for use as a standard assay to determine the activity of MerA and YpdA, primarily based on it showing lower errors overall and on the higher consistency in the raw data obtained.

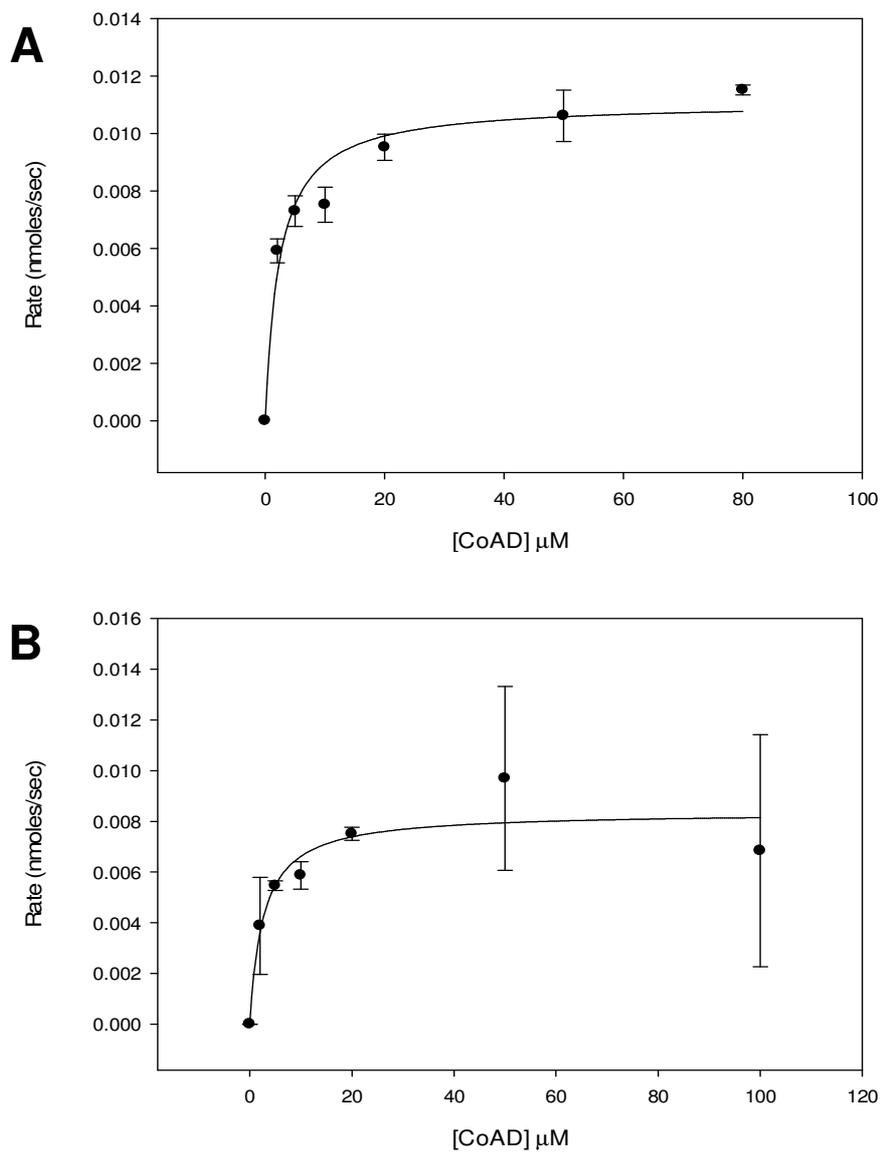


Figure 3.8: A comparison of the steady-state kinetics of the two CoADR assay conditions. **A)** Potassium phosphate conditions. Under these conditions, the data obtained displayed fewer standard errors and proved more reproducible. **B)** HEPES conditions. Despite yielding a very similar profile, the data obtained under the HEPES conditions displayed greater errors. All experiments were performed in triplicate.

Table 3.2: Comparison of the kinetic parameters of the two CoADR assays

	K_M (μM)	V_{max} (nmols.s ⁻¹)	k_{cat} (s ⁻¹)
Potassium Phosphate	2.36 ± 0.32	0.011 ± 0.0003	2.75
HEPES	2.63 ± 1.46	0.008 ± 0.0009	2

3.2.8 Catalytic activity of MerA

The characterisation of MerA was designed to explore two main ideas:

1. The protein was hypothesised to act as a secondary LMW thiol disulfide reductase during oxidative stress, in which case it may be able to reduce CoAD, and/or BSSB.
2. The homology of MerA to that of known mercuric reductases required that it be tested for potential mercuric ion reductase activity, even if this activity cannot be relevant in the context of redox balance maintenance. If the protein was found to reduce mercuric ions, then a working theory had to be developed as to the use of this function towards redox balance.

The following assays were therefore conducted to provide evidence in support or against these ideas.

3.2.8.1 MerA activity towards common LMW thiol disulfides

MerA was initially tested with the disulfides of known LMW thiols of *S. aureus* to determine its ability to reduce these. Using the phosphate buffer conditions described above, MerA was tested with CoAD and BSSB, as well as the CoA precursor, pantethine. The enzyme CoADR was assayed with CoAD under identical conditions, acting as a positive control to indicate the reduction of a disulfide (Figure 3.9). The results showed that MerA has no disulfide reductase activity with any of the substrates tested.

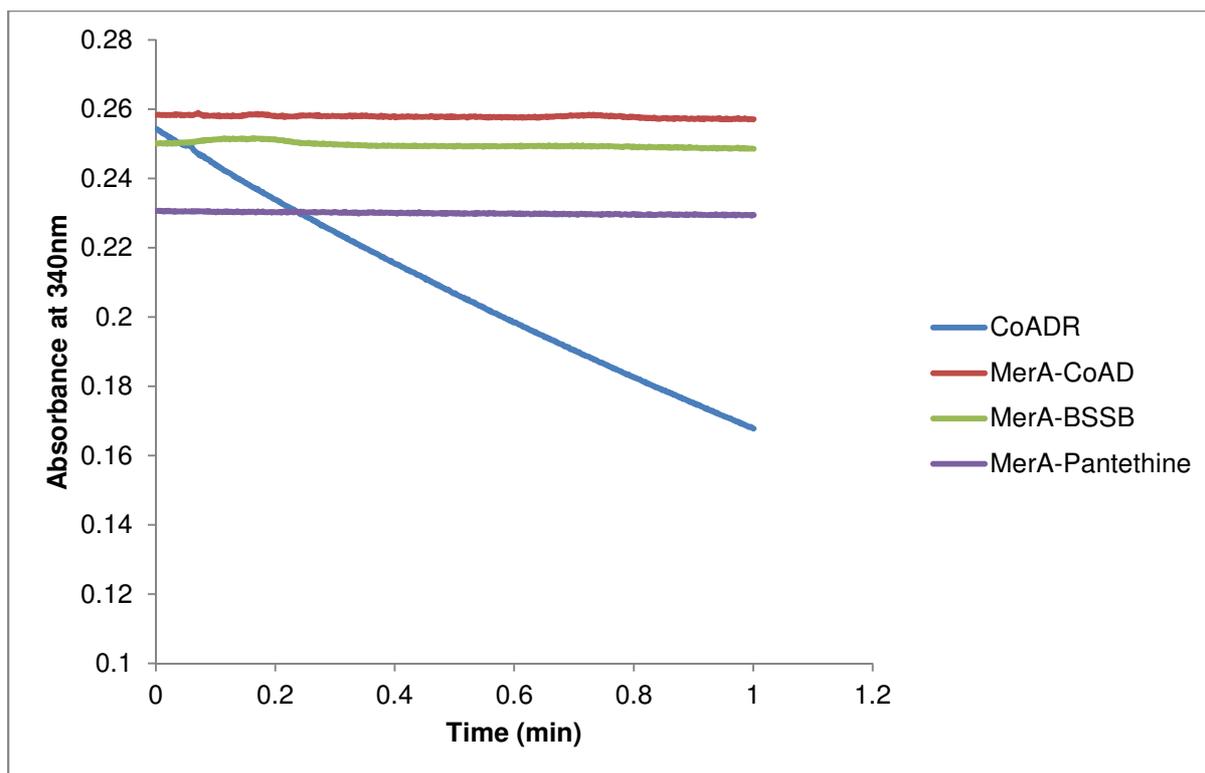


Figure 3.9: Catalytic activity of MerA towards various disulfides as monitored at 340nm. MerA was unable to reduce the disulfides CoAD, BSSB or pantethine. CoADR was assayed with CoAD under the same conditions, acting as a positive control for disulfide reductase activity.

3.2.8.2 MerA activity as a mercuric ion reductase

The routine mercuric reductase activity assay, described by Walsh *et al.*, was used to confirm whether or not the MerA homolog was a functional mercuric ion reductase.⁷ Known mercuric ion reductase enzymes catalyse the reduction of Hg^{2+} in a NADPH-dependent manner. Thus the reaction progress may be followed spectrophotometrically by monitoring the decrease in absorbance of NADPH at 340 nm. The MerA homolog of *S. aureus* was found to reduce Hg^{2+} in the form of HgCl_2 , giving the activity profile shown in Figure 3.10. Using this data and the Michaelis-Menten equation, the parameters in Table 3.3 were determined. In comparison to the K_m values determined for known mercuric ion reductases, the K_m for MerA and HgCl_2 is much higher. This suggests that Hg^{2+} may not be the real substrate for MerA.

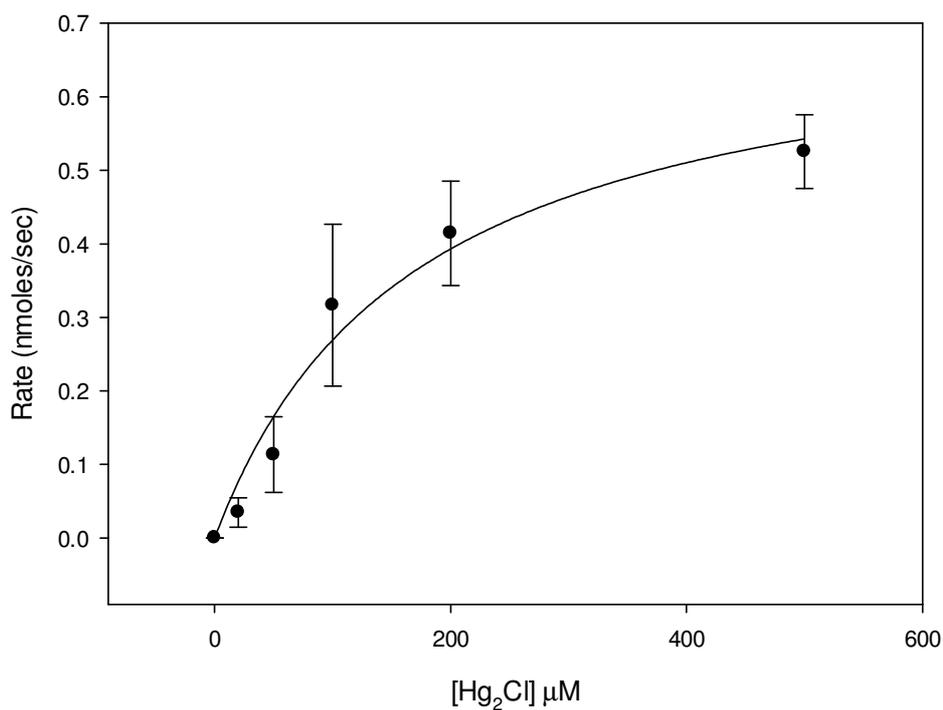


Figure 3.10: Steady-state kinetic profile of MerA with HgCl₂.

Table 3.3: Comparison of the kinetic parameters of MerA with that of known mercuric ion reductases.

Mercuric reductases with HgCl ₂	K_M (μM)	V_{max} (nmols.s ⁻¹)	k_{cat} (s ⁻¹)
MerA	169 ± 50.5	0.727 ± 0.093	0.74
Mercuric reductase <i>P. aeruginosa</i> ⁷	12	-	-
Mercuric reductase <i>E. coli</i> W224 ⁷	20	-	-

3.2.8.3 MerA as a potential ferric ion reductase

MerA's ability to reduce Hg^{2+} , albeit poorly, suggested that it had a latent activity towards the reduction of metals. In the context of the bacterial cell's redox chemistry, the most important metal is iron, as oxidative damage will convert all the Fe^{2+} found as enzyme cofactors (or part of enzyme cofactors, such as the iron sulphur clusters) to Fe^{3+} .¹ MerA was therefore assayed with nitrilotriacetic acid-Fe(III) (FeNTA) in order to assess the protein's ability to reduce Fe^{3+} (Figure 3.11). FeNTA was prepared according to the protocol laid out by Bunescu *et al.*⁶ MerA was assayed with FeNTA using the same protocol as described for the MerA- HgCl_2 assay³.

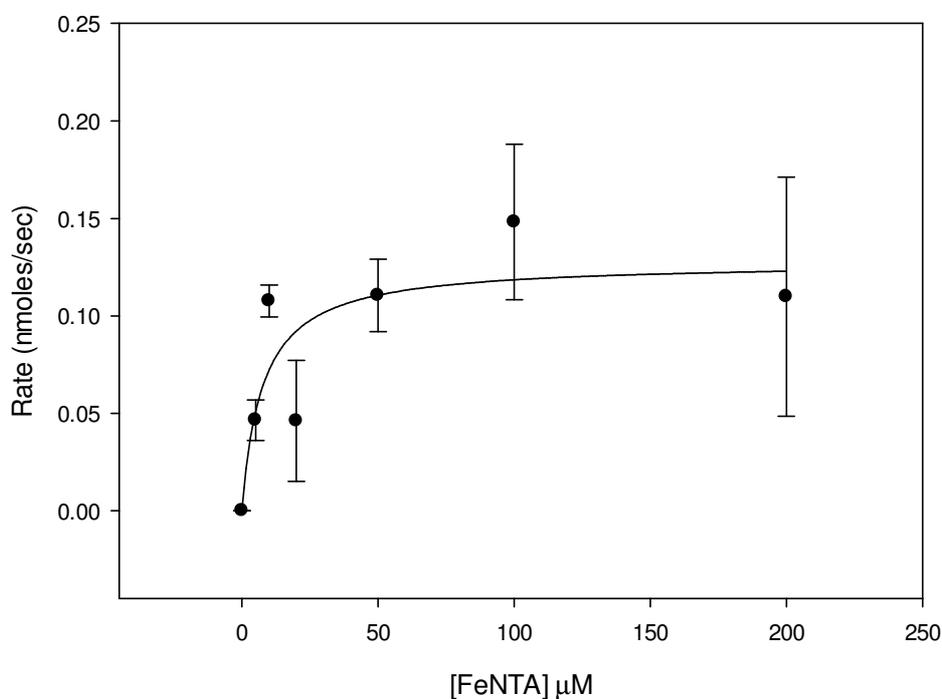


Figure 3.11: Steady-state kinetic profile of MerA with FeNTA.

Using this kinetic profile, kinetic parameters were determined (Table 3.4). These show that MerA has a far greater affinity for Fe^{3+} (in FeNTA) than for Hg^{2+} (in HgCl_2), indicating that the former is a more likely substrate for the protein. Moreover, the K_M observed for MerA with Fe^{3+} is comparable with that of characterised ferric ion reductases, although it has a much lower k_{cat} compared to these enzymes. This is a further indication that Fe^{3+} may be the preferred substrate of MerA. Conversely, MerA's V_{max} for the FeNTA reaction is much lower than that observed of HgCl_2 . Thus, while MerA has a much higher affinity for Fe^{3+} as a substrate, the reaction will proceed at a much lower rate at saturating conditions.

Table 3.4: Comparison of the kinetic parameters of MerA and known ferric reductases acting upon Fe³⁺.

Protein Organism	K_M (μM)	V_{max} (nmols.s ⁻¹)	k_{cat} (s ⁻¹)
MerA <i>S. aureus</i>	7.66 ± 4.52	0.127 ± 0.017	0.732
FerA <i>P. denitrificans</i> ²¹	5.5 ± 0.7	-	14 ± 1
FerA <i>R. sphaeroides</i> ²¹	4.2 ± 0.2	-	-

3.2.9 Catalytic activity of YpdA

The characterisation of YpdA's putative activity centred around three main concepts:

1. YpdA has been linked to BSH-related functions, and therefore its activity towards BSSB needed to be assessed. At the same time YpdA's activity towards other *S. aureus* biothiols could also be explored in order to rule out potential substrate promiscuity.
2. YpdA has also been linked to bacilliredoxin (a TFP) activity in the thiol-disulfide interchange system. Since no bacilliredoxin has been characterized in *S. aureus* as yet (two putative bacilliredoxins, YphP and YtxJ, have been proposed to act in *Bacillus subtilis*), YpdA's activity with a generic TFP such as TrxA could be tested.

3.2.9.1 Catalytic activity of YpdA towards common LMW thiol disulfides

YpdA was initially tested with the known LMW thiols of *S. aureus* to determine its ability to reduce one or more of these. Under the standard conditions determined previously, YpdA was tested with CoAD and BSSB, as well as the CoA precursor, pantethine. The enzyme CoADR was assayed with CoAD under identical conditions, acting as a positive control. Referring to Figure 3.12, the reaction of CoADR with CoAD shows a rapid decrease in the NADPH concentration, clearly indicating that the disulfide substrate is being reduced in a NADPH-dependent manner. Conversely, YpdA demonstrates no measurable reduction of the disulfide substrates, as the NADPH content remains constant throughout the assay.

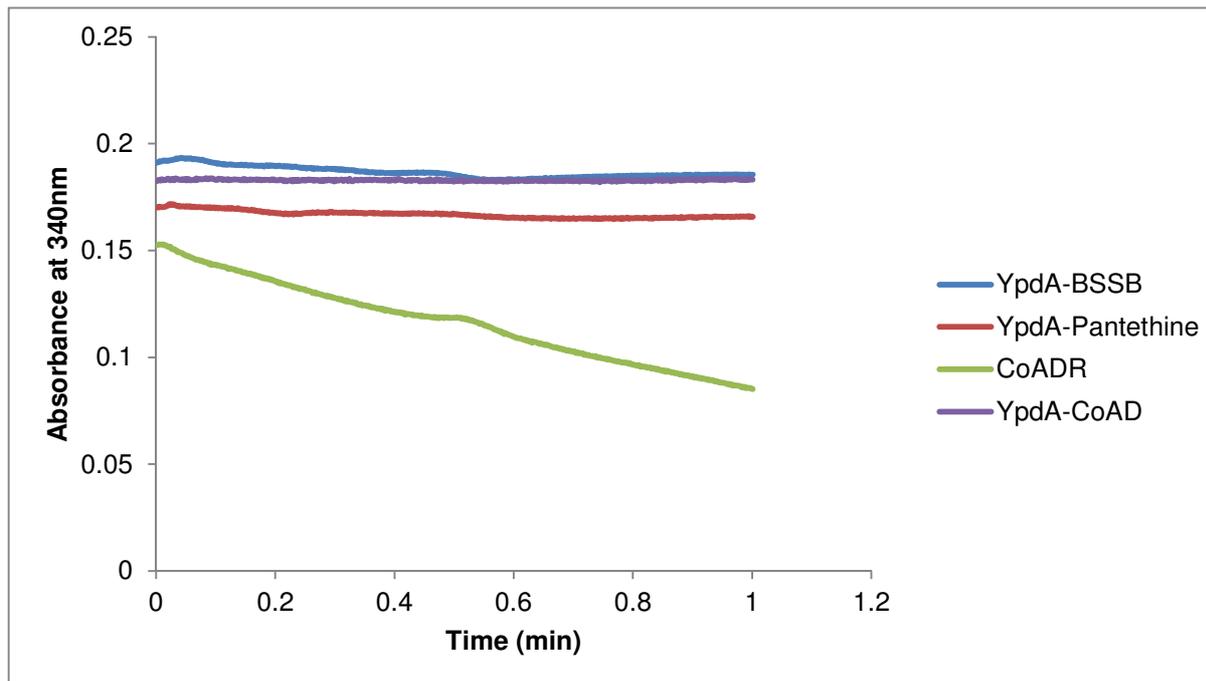


Figure 3.12: Catalytic activity of YpdA towards various disulfides as monitored at 340nm. YpdA was unable to reduce the disulfides CoAD, BSSB or pantethine. CoADR was assayed with CoAD under the same conditions, acting as a positive control for disulfide reductase activity.

3.2.9.2 YpdA as putative thioredoxin reductase

The sequence similarities between YpdA and known TrxR proteins suggest that it may require a corresponding TFP protein in order to function. Putative bacilliredoxins have been identified (for example YphP and YtxJ, see Chapter 2), and are suggested to fill this role.² However, since these proteins were not available in our lab, a more generic alternative was employed to test YpdA's potential to act as a TrxR protein. YpdA was thus assayed for enzymatic activity using TrxA as an interacting partner, and with DTNB as a substrate.

DTNB, or Ellman's reagent, is commonly used in assays to test thioredoxin activity, as a functional TrxR and Trx pair can reduce the compound's disulfide bond. The reduction of this bond leads to the formation of 2-nitro-5-thiobenzoate (NTB²⁻), a bright yellow compound, the formation of which may be monitored at 412 nm.¹¹

The reduction of DTNB via the thioredoxin system proteins, TrxB and TrxA, was used as a positive control for the experiment (Figure 3.13). The reaction of TrxB and TrxA with DTNB

shows the clear increase of NTB^{2-} concentration over the duration of the assay. In contrast, negative controls, whereby either TrxA or TrxB have been eliminated from the reaction, show no quantifiable NTB^{2-} formation. Assaying YpdA with TrxA demonstrated no discernible NTB^{2-} formation. This indicates that the proteins did not interact in a manner similar to that of known thioredoxin system proteins. This could be the result of one of two situations: Either YpdA is not capable of acting on TrxA as a substrate (it is not its cognate TFP partner), or YpdA is not a functional TrxR at all.

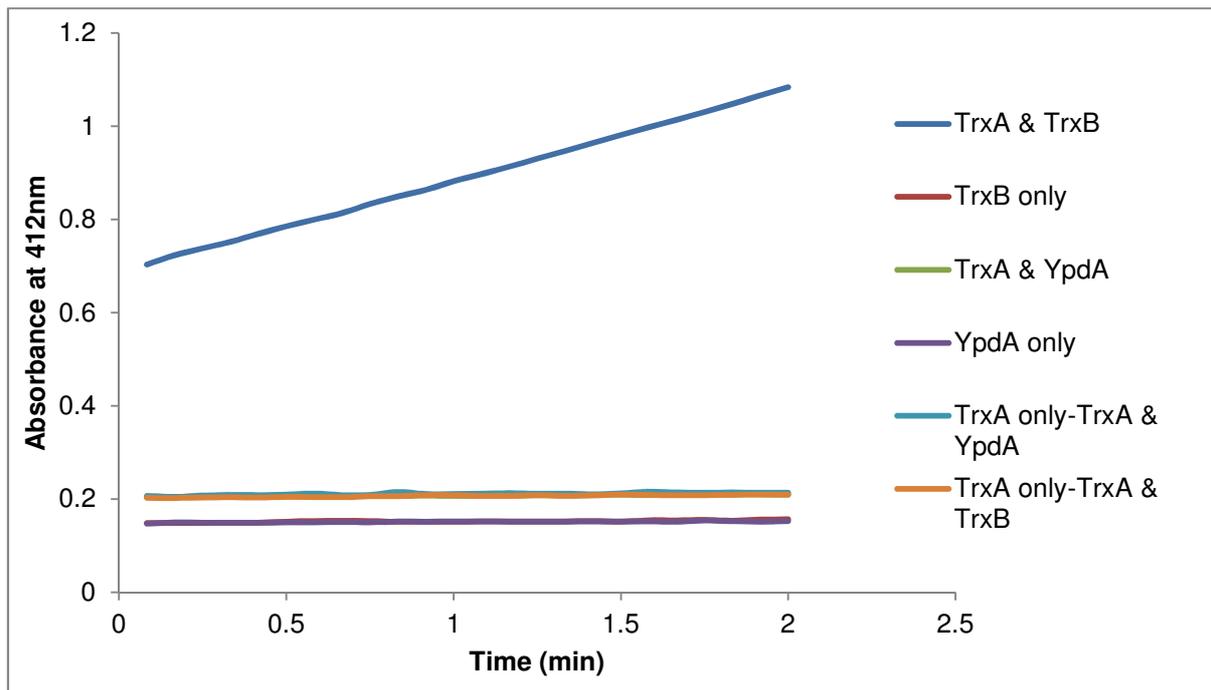


Figure 3.13: Interaction between YpdA and TrxA, using DTNB as a substrate. YpdA was assayed with DTNB as a substrate. TrxA was supplied as an interacting TFP partner. The same assay was conducted with TrxB and TrxA as a positive control. Negative controls were as follows: TrxA-YpdA assay, omitting TrxA; TrxA-YpdA assay, omitting YpdA; TrxB-TrxA assay, omitting TrxA; TrxB-TrxA assay, omitting TrxB.

3.3 Discussion

3.3.1 Activity characterization of MerA

Known mercuric ion reductases require NADPH for enzymatic activity and do not utilise NADH.^{3, 4} The titration of MerA with NADPH and NADH mirrors the reduction reactions expected from both functional mercuric ion reductases and most disulfide oxidoreductases. While this result does not confirm MerA as either a mercuric ion or LMW thiol-disulfide reductase, it did clearly establish the cofactor requirements of the protein, and could be utilised for further experimentation.

A BLAST search using the MerA amino acid sequence as query reveals a high sequence similarity between MerA and known glutathione reductases. This in itself is not surprising, since both the mercuric ion and glutathione reductase enzymes are flavoproteins belonging to the DSR subgroup of FDRs.⁸ Additionally, a study by Walsh *et al.*^{7, 8} found that the two enzymes also share extensive homology with regards to their active site sequences, as well as their mechanism of action towards substrates. Therefore, the initial hypothesis that MerA could be involved with the reduction of LMW disulfides in *S. aureus* was prompted by the similarities between MerA and glutathione reductase. However, MerA was not found to be able to reduce any of the disulfides in the assay conditions that were used. The results obtained from these assays thus indicate that MerA does not act as a LMW disulfide reductase.

The results from the bioinformatic analysis initially made it seem unlikely that MerA could as a functional mercuric ion reductase, based on the lack of a pair of Cys residues at its C-terminal. Thus it was surprising to observe the quantifiable reduction of Hg^{2+} by MerA. The calculated K_m value for MerA with HgCl_2 was compared to that of three other bacterial mercuric reductases (Table 3.3). This revealed that MerA had a much lower affinity for HgCl_2 than that of the known mercuric reductases.³ This lowered substrate affinity in MerA may be due to its lack of the C-terminal cysteine pair, as these Cys residues are suggested to assist the protein in acquiring Hg^{2+} from its environment.^{3, 4}

The successful demonstration of mercuric ion reductase activity in MerA made the protein's role in oxidative stress resistance even more puzzling. Although undeniably important to a cell experiencing heavy metal stress, the link between Hg^{2+} reduction and oxidative stress resistance was unclear. This led to the formation of a new hypothesis: was the protein capable of reducing another biologically-relevant metal, namely iron? Iron-derived ions such as Fe^{2+} are critical in cellular function, as they often act as enzyme cofactors essential to catalysis, e.g. heme, iron-sulfur clusters, etc.¹⁶ The results of the assay were clear: MerA

was found to not only reduce Fe^{3+} in the form of an NTA complex, but displayed a higher affinity for the ion compared to Hg^{2+} . Whether or not the protein's ability to reduce Fe^{3+} is relevant to the organism's oxidative stress defense mechanisms requires further investigation, especially since the NTA counter-ion is not biologically relevant. However, this study has proposed a tentative hypothesis for the Fe^{3+} -reducing abilities of MerA during oxidative stress, namely the reactivation of oxidised 4[Fe-S] enzymes.

4[Fe-S] proteins are heavily involved in a number of crucial cellular processes, most notably electron transport.⁹ The inactivation of these proteins for whatever reason would thus have disastrous implications for a living cell. During an oxidative blast, O_2^- can react with the 4[Fe-S] clusters of proteins possessing such groups, creating inactive Fe (IV) enzymes and often leading to the release of Fe^{3+} ions from the enzyme.^{5,9,24} However, these proteins may be re-activated in the presence of free Fe^{2+} ions and thiols.²⁴ At this point, MerA could potentially assist in the reactivation of the oxidised 4[Fe-S] by reducing Fe^{3+} to Fe^{2+} . The inactivated proteins could thus be restored to normal function. Furthermore, whilst *S. aureus* possesses membrane-bound ferric ion transporters for iron assimilation, it does not have native cytoplasmic ferric reductases.²¹ This leaves a niche in the *S. aureus* proteomic network for a ferric reductase during oxidative stress. The exploration of this hypothesis as a function of MerA may thus be investigated in future studies.

3.3.2 Activity characterization of YpdA

Gaballa *et al.* were the first to draw attention to YpdA, identifying it as a FDR with a potential role in BSH-dependent processes in *B. subtilis*.¹² Flagged by the EMBL STRING programmes, the *ypdA* gene was one of four found to occur with high frequency in the genomes of BSH-synthesising bacteria. Bioinformatic studies of the *S. aureus* genome later revealed that YpdA bears high sequence homology to known TrxR proteins, and falls within the same FDR subgroup: the alkyl hydroperoxide reductases¹²

The similarities displayed by YpdA to TrxR suggest that it is likely to require a corresponding TFP (most likely a thioredoxin) in order to carry out its functions. This led to the conclusion that YpdA could be responsible for the reduction of oxidized bacilliredoxin proteins. Moreover, it has also been proposed as a candidate for the as yet unidentified BSSB reductase.

The sequence analysis of YpdA revealed several deviations from that of known TrxR proteins. Most glaringly was the lack of an active-site Cys pair in a CxxC motif. This motif is highly conserved amongst TrxR proteins, as it is critical to catalytic function.¹¹ Additionally,

the NADPH-binding site of YpdA contains a sequence insert, as well as several changes in amino acid residues. Although this potentially indicated that the protein would not be able to bind NADPH, this was disproven; anaerobic titrations of YpdA with NADPH demonstrated effective binding and reduction of the protein.

A study by Hernandez *et al.* attempting to characterise a putative TrxR prompted reconsideration regarding the cofactor requirements of YpdA.¹³ Hernandez found that the putative TrxR utilised neither NADPH nor NADH as cofactor, relying instead on an unknown electron donor.¹³ It was thus deemed prudent to determine the preferred cofactor of YpdA, if any, prior to commencing assays.

Under standard conditions, YpdA exhibited no activity with any of the disulfides supplied, including BSSB. This result was not unexpected; if YpdA is in fact a TrxR-like protein, then it would be incapable of reducing disulfide bonds in the absence of an interacting TFP partner. This result did, however, rule YpdA out as a direct BSSB reductase.

Research into the BSH-related pathways has identified two putative bacilliredoxins: YphP and YtxJ (see Chapter 2). These proteins have been suggested as potential interacting partners for YpdA, and as such must be tested with the latter in order to ascertain such a hypothesis. However, neither protein was cloned and purified as part of this study, although this clearly needs to form part of future work. Thus, in the absence of these putative partners, YpdA was assayed with TrxA as a substitute. The inability of YpdA to reduce DTNB in the presence of TrxA thus leads to two different conclusions. First, YpdA could be incapable of reducing DTNB with any TFP partner other than its own. Alternatively, YpdA simply is not a functional TrxR. When revisiting the bioinformatic analysis of YpdA, the protein's lack of a redox-active cysteine pair does suggest that it may not be capable of catalysing the reduction of any disulfide. However, a final conclusion regarding YpdA cannot be drawn without an attempt to identify any interacting protein partners. Such studies will thus undoubtedly form part of a future investigation into the protein's function.

3.4 Conclusion

3.4.1 MerA

The MerA protein was originally deemed incapable of mercuric ion reductase activity due to its missing penultimate Cys-pair and other sequence variations from the known. Despite these anomalies, MerA was proven capable of reducing Hg^{2+} ions in a NADPH-dependant manner. The protein demonstrated a much lower affinity for HgCl_2 compared to that of characterised mercuric ion reductases, averaging a K_M of 169 μM compared to their reported

K_M values of 12-20 μM .⁷ This is potentially attributed to the missing C-terminal Cys-pair, which is implicated in the acquisition of Hg^{2+} for catalysis.

The reduction of Fe^{3+} by MerA was shown to yield a significantly lower K_m in the presence of Fe^{3+} compared to Hg^{2+} , indicating that the former is a more likely substrate for the protein. The results of this study therefore proposes that MerA may reduce Fe^{3+} during oxidative stress, thus providing Fe^{2+} for the reactivation of oxidised 4[Fe-S] cluster proteins.

3.4.2 YpdA

Despite much sequence identity with a functional TrxR protein, YpdA lacks the critical active site CxxC motif for disulfide reductase function. Furthermore, the bioinformatic analysis revealed an insert and various amino acid residue discrepancies in the NADPH-binding domain of YpdA. This does not appear to affect the protein's ability to bind NADPH however, as anaerobic titrations of YpdA with NADPH revealed rapid and effective reduction of the FAD cofactor.

Under standard conditions, YpdA was incapable of reducing CoAD, BSSB or pantethine. Initially, this was attributed to lack of a corresponding TFP, as known TrxR proteins require an interacting TFP for catalytic function. TrxA was thus provided as a TFP for YpdA in order to test the latter's ability to interact with a generic Trx protein. Assaying YpdA with TrxA and DTNB revealed no quantifiable activity. This could be the result of two possible situations: Either YpdA requires its specific TFP for activity and is unable to interact with TrxA, or YpdA is an inactive TrxR homolog possessing no disulfide reductase activity. A thorough protein-protein interaction study with YpdA will be required by future studies in order to establish whether or not YpdA is in fact capable of enzymatic activity.

3.5 Experimental

3.5.1 Materials

All buffer components and other chemicals were obtained from Sigma-Aldrich. BSSB was generously provided by Dr. Chris Hamilton of the University of East Anglia. CoAD was synthesised by Bertus Moolman at the University of Stellenbosch. Restriction enzymes were purchased from Fermentas. All primers used were synthesised by Inqaba Biotec in Pretoria.

3.5.2 DNA amplification

3.5.2.1 *merA*

The *merA* gene (SAOUHSC_00581) was amplified from genomic *Staphylococcus aureus* DNA strain RN4220 (isolated using a genomic DNA extraction kit from Novagen) using the following primers: Forward: 5'-GCAGTCACTGTCACCATGG TCGTAATATTG -3' and reverse: 3'-CACCAGGTTTTTATGTTTTCTCGAGGAAATTAATAA -5'. With these primers an NcoI (underlined) and an XhoI (underlined) restriction enzyme site are introduced in the forward primer and the reverse primer respectively. The amplification mixture was set up as follows: Tubes 1 to 3 contained Pfu buffer, 0.4 mM dNTP mix, 1 mM forward primer, 1 mM reverse primer, 1 ng genomic DNA, 1.25 U Pfu DNA polymerase and distilled, deionized water (ddH₂O) to a final volume of 25 µl. Tubes 4 to 6 contained all of the above components, with only the substitution of ddH₂O for a 1.7 M solution of betaine monohydrate, bringing the final concentration of betaine monohydrate to 1 M in each mixture. The PCR program had an initial step of 2 min at 94°C followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s and polymerization for 1 min at 70°C. The reaction mixtures were subjected to gel electrophoresis on a 1% agarose gel and subsequently visualized through gel staining with SYBR™ gold (Invitrogen) and viewed on a Darkreader. The best product bands were seen at an MgSO₄ concentration of 3 mM and 4 mM. The product bands were excised and purified using the Novagen gel clean-up kit. These PCR products were used in the construction of the pET28a-*merA* plasmids.

3.5.2.2 *ypdA*

The *ypdA* gene (SAOUHSC_01499) was amplified from genomic *Staphylococcus aureus* DNA strain RN4220 using the following primers: Forward: 5'-GAGGCCGAACCATATGCAAAAAGTTGAAAGT-3' and reverse: 5'-GTACATAGACCTCTCGAGTTATGATTCTAAGGG-3'. With these primers an NdeI (underlined) and an XhoI (underlined) restriction enzyme site are introduced in the forward primer and the reverse primer respectively. Amplification of the *ypdA* gene was performed according to the procedure previously discussed. Following amplification, the reaction mixtures were visualised and the product band purified as previously outlined. Optimum results were obtained at an MgSO₄ concentration of 4 mM.

3.5.3 Restriction digests

Restriction digests were carried out on the *merA* PCR product and pET28a(+) using NcoI and XhoI restriction enzymes (Fermentas). The pET28a(+) plasmids were isolated from *E. coli* using the Zippy® plasmid preparation kit (Fermentas). The digestions were performed using Fermentas FastDigest buffer, 16 µL PCR product/plasmid and 10 U each NcoI and XhoI. These reactions were incubated at 37°C for 1 hour prior to 1% agarose gel

electrophoresis. Bands of the correct size were excised and purified using the Novagen gel clean-up system. Restriction digests of the *ypdA* PCR product and pET28a(+) were carried out using NdeI and XhoI (Fermentas) in the same procedure as described above.

3.5.4 Plasmid construction

The pET28a(+) plasmid system was used for the construction of all expression vectors required for this study. The molar concentrations of both the gene inserts and isolated plasmids were obtained using the Qubit® Fluorometric Quantitation system (Life Technologies). Equal molar amounts of the restriction digested plasmid and PCR product were added together in a volume of 5 µL. A final volume of 10.5 µL was obtained by adding Quick Ligation Buffer and 10 U of Quick T4 DNA Ligase (both purchased from New England Biolabs) to the reaction mixture. Following incubation for 5 minutes at room temperature, the mixture was transformed into competent *E. coli* Mach1 cells. Transformation was performed as follows: 1 µL of the plasmid-insert construct was added to 80 µL of chemically-competent Mach 1 cells, and incubated on ice for 30 mins. The cell mixture was heat-shocked at 42°C for 45 seconds, and then cooled on ice for 5 mins. The cell mix was then incubated at 37°C for 1 hour before being centrifuged at 8000 RPM for 4 mins. Half of the supernatant was discarded, with the cell pellet being re-suspended in the remaining supernatant. The re-suspended cells were then plated onto LB plates containing 30 mg/L kanamycin and incubated overnight at 37°C.

Colonies were screened for the *merA* and *ypdA* gene inserts by lysis in gel loading buffer and direct electrophoresis on a pre-stained 1% agarose gel, using the parent pET28a(+) plasmid as a reference. Only plasmids that appeared larger than the pET28a(+) were selected for further screening. The corresponding colonies of the chosen plasmids were cultured overnight in LB media containing 30 mg/L kanamycin at 37°C. Plasmids from these cultures were purified using the Zippy Plasmid Miniprep I Kit (Zymo Research) and subjected to a restriction digest reaction with the appropriate restriction enzymes. The restriction digestion reactions were subsequently visualised on a pre-stained 1% agarose gel to determine the correct banding patterns. The plasmid was sequenced and, if construction was successful, the plasmid was stocked for future use.

3.5.5 Protein expression and purification

Protein expression for all targets was first conducted in small scale in order to determine optimum conditions for soluble expression of the proteins of interest. Following elucidation of these conditions, large scale expression of target proteins was performed. All growth media

and plates used in the expression of the target proteins contained the antibiotic kanamycin (30 mg/L).

3.5.5.1 Expression trials

An expression plasmid was selected from those constructed earlier and transformed into *E. coli* BL21*(DE3) cells. An overnight culture was grown from a single colony of the transformed cells in preparation for expression trials. All expression trials were conducted in 15 mL Corning® centrifuge tubes. Each tube contained 4 mL LB media and 100 µL overnight culture, forming an expression culture. Expression cultures were incubated at 37°C with shaking until an OD₆₀₀ reading of 0.6 was reached, at which point IPTG was added from a 10 mM stock. Following the addition of IPTG, the cultures were topped up with LB media to give a final volume of 5 mL. The following parameters were varied in order to determine the optimum environment for soluble protein expression:

- The final IPTG concentration in each tube: 1 mM, 0.5 mM, 0.2 mM and 0.05 mM.
- The temperature at which expression took place: 22°C or 37°C.

Following overnight expression, the cells were harvested via centrifugation and either processed immediately or stored at -20°C for use at a later opportunity.

3.5.5.2 Large scale expression

LB media (500 mL) was inoculated with 2 mL of an overnight culture of *E. coli* BL21*(DE3) containing the desired expression plasmid. The culture was incubated on an orbital shaker at 37°C until an OD₆₀₀ of 0.5-0.6 was reached. The optimum amount of IPTG (pre-determined from small scale trials) was added and the culture was further incubated at the similarly determined temperature overnight. Cells were then collected by centrifugation at 4500×g for 30 minutes at 4°C. As with small scale expressions, the pellets obtained were either processed directly after collection or stored at -20°C until required.

3.5.5.3 Analysis of expression trials

Cell pellets obtained from small scale expression trials were re-suspended in 300 µL BugBuster® protein extraction solution (Novagen) and incubated on a shaking platform for 20 minutes at room temperature. The reaction mixture was centrifuged at 1300×g for 20 minutes, with the supernatant subsequently transferred to a clean tube. The cell debris pellet was re-suspended in 100 µL of 10 mM Tris-HCl, pH 8.0. 15 µL of both the supernatant and

re-suspended cell pellets were analysed on a 12% SDS-PAGE gel for soluble and insoluble proteins respectively.

3.5.5.4 Purification of large scale protein expression

The cell pellets obtained from large scale expression were re-suspended in binding buffer (20 mM Tris-HCl, 5 mM imidazole, 500 mM NaCl, pH 7.0), in a volume equating to 10 mL per 1 g of pellet. Cells were lysed via sonication, and the cell debris removed by centrifugation at 1500×g for 30 minutes at 4°C. The supernatant was filtered with a Pall Acrodisc PSF GxP/GHP 0.45 µm filter prior to injection into the ÄKTApriime system (Amersham Biosciences).

All proteins were purified using a 1 mL HiTrap Chelating HP column. Prior to loading the protein, the column was equilibrated with 10 column volumes of H₂O and 5 column volumes of binding buffer (20 mM Tris-HCl, 5 mM imidazole, 500 mM NaCl, pH 7). The protein was loaded and followed by two wash steps: 10 column volumes of binding buffer, followed by another 5 column volumes containing 15% elution buffer (20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 7.9). This second wash step was essential for eliminating any non-specifically bound proteins. The recombinant MerA-His protein was eluted over the course of 10 column volumes of 100% elution buffer. For the duration of the purification, the flow rate was maintained at 1 mL/min, whilst protein elution profiles were monitored at 254 nm. All columns used were purchased from Amersham Biosciences.

3.5.5.5 SDS-PAGE gel analysis

Proteins obtained from both small and large scale expression were visualised by SDS-PAGE using 12% gels. An equal volume of 2× SDS-PAGE loading buffer (0.125 M Tris-HCl, 4% SDS, 30% glycerol, 1.5% β-mercaptoethanol and bromophenol blue, pH 6.8) was added to the protein samples and the reaction mixtures were incubated at 95°C for 5 minutes. 15 µL of all samples were loaded onto the gel and electrophoresed in SDS-PAGE running buffer (0.1% SDS, 0.025 M Tris and 0.192 M glycine). Completed gels were stained with Coomassie blue stain (45 mL MeOH, 40 mL ddH₂O, 10 mL acetic acid and 250 mg Coomassie Brilliant Blue) for 30 minutes and later de-stained for 2 hours using destain solution (45 mL MeOH, 40 mL ddH₂O, 10 mL acetic acid).

3.5.5.6 Protein concentration determination

All protein concentration was determined using the Bradford protein assay. A standard curve of absorbance versus concentration was plotted with the use of BSA standards (Biorad). All protein samples and standards were treated with Coomassie Brilliant Blue and incubated at room temperature for 5-10 minutes. Absorbance was subsequently measured at 595 nm using a Thermo Varioskan multiplate spectrometer in 96-well plates.

3.5.6 Enzymology

Unless otherwise stated, all assays performed were done in triplicate.

3.5.6.1 Anaerobic titration with NADPH/NADH¹³

YpdA was titrated with NADPH (0.5 – 5.0 mol NADPH per mol FAD) under anaerobic conditions at 25^oC in 50 mM Tris-HCl (pH 7.5), and 150 mM NaCl. The titration was conducted in an anaerobic cuvette, sealed with a gas-tight rubber septum and repeatedly flushed with nitrogen gas. All reactants were flushed with nitrogen gas prior to titration. The reduction of the enzyme flavin by NADPH was monitored by collecting spectra from 800 nm to 240 nm. The anaerobic titration of TrxB with NADPH and NADH was used as a control. This process was then repeated with YpdA and NADH, as well as MerA with NADPH and NADH.

3.5.6.2 Standard assay conditions: Potassium phosphate conditions¹⁷

A master mix was made up containing 50 mM potassium phosphate (pH 7.0), 0.5 mM EDTA, 35.56 μ M NADPH and 22.22 nM of the CoADR enzyme. Substrate solutions of CoAD were prepared separately. The concentrations varied such that the final CoAD concentrations fell between 0 – 60 μ M. All solutions were incubated for 5 min at 25 °C before the assay. The reaction was initiated by adding 180 μ L of the master mix to 20 μ L of the substrate sample within a 96-well plate, creating a final volume of 200 μ L. The decrease in absorbance at 340 nm was followed for 2 min at 25^oC in a Thermo Varioskan multiplate spectrometer.

3.5.6.3 Standard assay conditions: HEPES conditions¹⁷

A master mix was prepared containing 40 mM HEPES (pH 7.5), 1.0 mM EDTA, 166.67 μ M NADPH and 22 nM CoADR enzyme. Substrate solutions of CoAD were prepared separately. The concentrations varied such that the final CoAD concentrations fell between 0 – 60 μ M. All solutions were incubated for 5 min at 37 °C before the assay. The reaction was initiated by adding 225 μ L of the master mix to 25 μ L of the substrate sample within a 96-well plate,

creating a final volume of 250 μL . The decrease in absorbance at 340 nm for each enzyme was followed for 10 min at 37°C in a Thermo Varioskan multiplate spectrometer.

3.5.6.4 Protein activity tests under standard conditions¹⁷

The ability of MerA and YpdA to reduce CoAD was assayed at 25 °C in a total volume of 180 μL . The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.5 mM EDTA and 32 μM NADPH. The final CoAD concentration was 60 μM . One unit of activity is defined as the amount of enzyme catalysing the oxidation of 1 μmol of NADPH (decrease in A_{340}) per minute at 25 °C.

A master mix was made up containing 50 mM potassium phosphate (pH 7.0), 0.5 mM EDTA, 35.56 μM NADPH and 22.22 nM of the particular enzyme being tested. A substrate solution of 600 μM CoAD was prepared separately, and all solutions were incubated for 5 min at 25 °C before the assay. The reaction was initiated by adding 180 μL of the master mix to 20 μL of the substrate sample, mixed briefly, and 180 μL of the reaction mixture transferred to a 1 cm pathlength cuvette. The decrease in absorbance at 340 nm was followed for 1 min at 25°C in an Agilent Cary 60 UV-Vis spectrometer. This procedure was repeated with the substrates BSSB and pantethine.

3.5.6.5 Mercuric reductase activity assay⁷

The mercuric reductase activity of MerA was assayed at 37°C in a total volume of 180 μL containing 80 mM sodium phosphate (pH 7.4), 200 μM NADPH, 100 μM HgCl_2 and 1 mM 2-mercaptoethanol.³

A master mix was prepared consisting of 88.89 mM sodium phosphate (pH 7.4), 222.22 μM NADPH, 111.11 μM HgCl_2 and 1.11 mM 2-mercaptoethanol. Both the master mix and a separate substrate solution of undiluted MerA protein were incubated at 37°C for 10 minutes prior to initiation. The reaction was initiated by adding 180 μL of the master mix to 20 μL of the MerA protein, mixing briefly and finally transferring 180 μL of the reaction mix to a 1 cm pathlength cuvette. The decrease in absorbance at 340 nm was followed for 2 min at 25°C in an Agilent Cary 60 UV-Vis spectrometer.

3.5.6.6 MerA Fe(III) reduction assay^{6,7, 14}

The activity of MerA towards Fe^{3+} was assayed at 37°C in a total volume of 180 μL containing 80 mM sodium phosphate (pH 7.4), 200 μM NADPH, 100 μM Fe(III)-NTA and 1 mM 2-mercaptoethanol.

Fe(III)-NTA was prepared as follows²: To a 100 mL aqueous solution containing 4 mM NTA at a pH 4.0 was added, under stirring, 100 mL of a freshly prepared solution of 4 mM Fe(ClO₄)₃. This mixture was stirred for 1 h, and the pH was adjusted to 4.0 with 1 M NaOH. The total complexation of the iron was controlled by UV-visible spectrophotometry ($\epsilon_{260} = 6,000 \text{ M}^{-1} \text{ cm}^{-1}$). The pH of the FeNTA solution was adjusted to 7.0 with 1 M NaOH just before the experiment.

A master mix was prepared consisting of 88.89 mM sodium phosphate (pH 7.4), 222.22 μM NADPH, 111.11 μM FeNTA and 1.11 mM 2-mercaptoethanol. Both the master mix and a separate substrate solution of undiluted MerA protein were incubated at 37°C for 10 minutes prior to initiation. The reaction was initiated by adding 180 μL of the master mix to 20 μL of the MerA protein, mixing briefly and finally transferring 180 μL of the reaction mix to a 1 cm pathlength cuvette. The decrease in absorbance at 340 nm was followed for 2 min at 25°C in an Agilent Cary 60 UV-Vis spectrometer.

3.5.6.7 YpdA-TrxA assay¹³

YpdA was assayed with DTNB using TrxA as an interacting protein partner. A master mix was prepared consisting of 58.82 μM TrxA, 17.6 nM YpdA and 225 μM NADPH. A substrate solution of 1 mM DTNB was prepared. All reagents were incubated at 37°C for 10 min prior to the assay. The reaction was initiated by adding 160 μL of master mix to 40 μL DTNB. The increase in absorbance at 412 nm was followed for 2 min at 37°C in a Thermo Varioskan multiplate spectrometer.

3.6 References

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Chapter 4

Cloning, expression, purification and activity analyses of the *S. aureus* TrxB and TrxA proteins

4.1 Introduction

Thioredoxin (Trx) and thioredoxin reductase (TrxR) are important disulfide reductases in most living cells. Collectively known as the thioredoxin system, Trx, TrxR and NADPH are capable of reducing a number of protein disulfide substrates, most notably peroxiredoxins, ribonucleotide reductases and methionine sulfoxide reductases.¹⁰ The importance of the thioredoxin system is further highlighted by the fact that TrxR proteins are essential to bacterial cells; any attempts to create *trxR*-null mutants prove lethal to cell survival.^{4,11}

The apparent non-essentiality of CoADR for *S. aureus* survival indicates that a second system exists that may reduce CoAD with comparable efficiency.⁴ The results of Chapter 3 have already indicated that neither MerA nor YpdA are likely to fulfil such a role. Thus the thioredoxin system, which is already a known and flexible disulfide-reducing system, would be the logical option for such a back-up. This study thus aims to explore the *S. aureus* thioredoxin system's activity towards LMW thiol disulfides in order to assess their role in oxidative stress.

This chapter describes the cloning, expression and purification of the *S. aureus* Trx and TrxR proteins (TrxA and TrxB respectively), as well as their activity towards LMW thiol disulfides.

4.2 Results

4.2.1 Gene amplification and plasmid construction

The *trxB* gene (SAOUHSC_00785) was amplified from genomic *Staphylococcus aureus* DNA strain RN4220. An N-terminal NdeI site and a C-terminal XhoI site were introduced to the gene during amplification, thus allowing *trxB* to be cloned into the pET28a(+) expression vector. The restriction sites introduced were chosen such that TrxB would be expressed with an N-terminal His-tag. The formation of a pET28a(+)-*trxB* hybrid plasmid was confirmed with digestion screening and sequencing of the constructed plasmid and insert..

The *trxA* gene (SAOUHSC_01100) was similarly inserted into the pET28a(+) expression vector to create the pET28a(+)-*trxA* hybrid plasmid.

4.2.2 Heterologous expression trials

Expression trials were carried out in order to elucidate the optimum expression parameters for soluble expression of TrxB. The trials were conducted using *E. coli* BL21* (DE3) cells and expression was allowed to continue overnight. Optimum expression was obtained at 37°C, with a total IPTG concentration of 0.5 mM, as can be seen from the SDS-PAGE analysis shown in Figure 4.1.

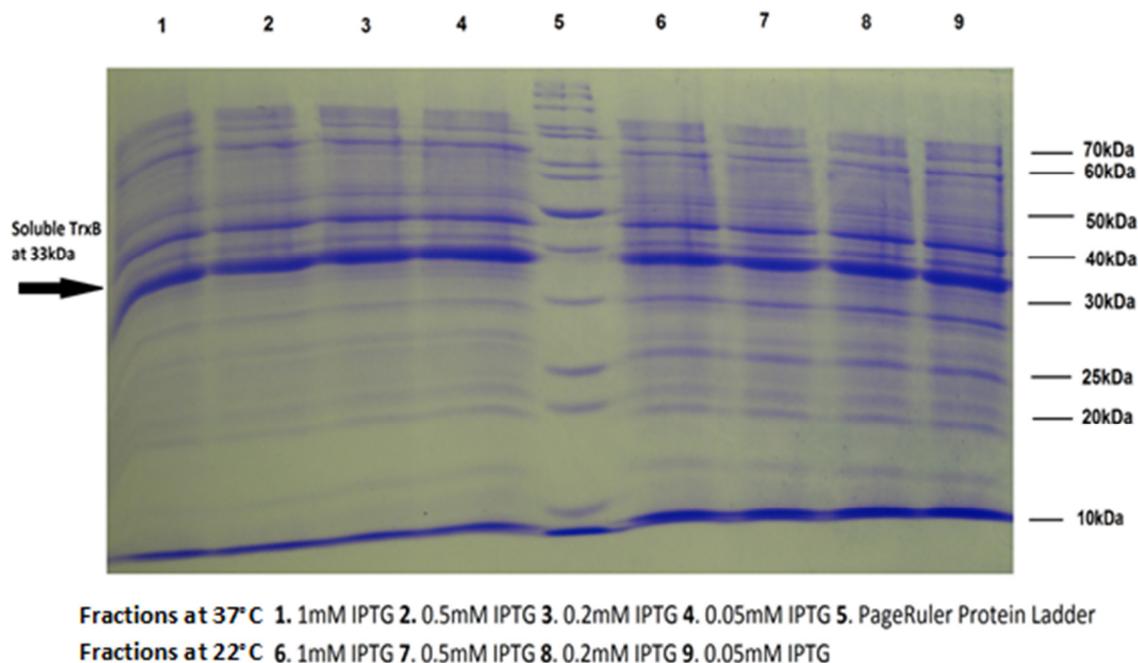


Figure 4.1: SDS-PAGE analysis of soluble expressed TrxB protein. The crude cell extracts from different expression conditions were separated by SDS-PAGE on a 12% gel. The TrxB protein is tentatively identified based on it having the expected size (as judged by comparison to the molecular weight markers) and is indicated by the arrow.

Expression trials for TrxA were carried out using the same protocol as described above for TrxB. Optimum expression was obtained at 22°C, with an IPTG concentration of 0.5 mM, as demonstrated by the SDS-PAGE analysis shown in Figure 4.2.

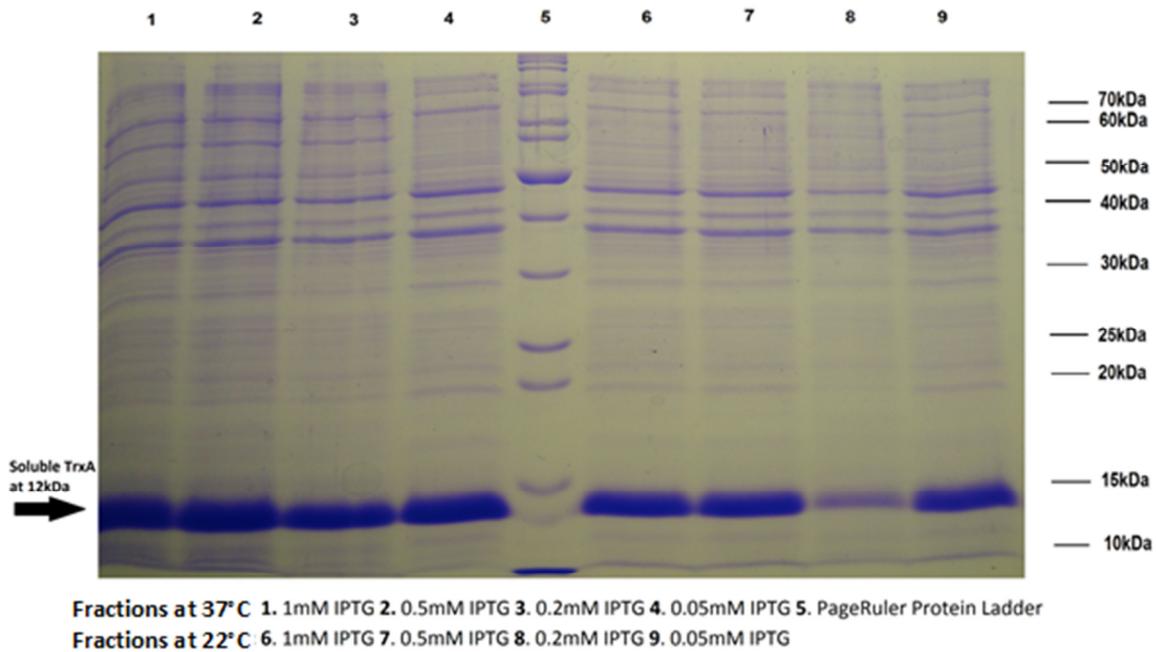


Figure 4.2: SDS-PAGE of soluble expressed TrxA protein. The crude cell extracts from different expression conditions were separated by SDS-PAGE on a 12% gel. The TrxA protein is tentatively identified based on it having the expected size (as judged by comparison to the molecular weight markers) and is indicated by the arrow.

4.2.3 Protein purification

The large-scale expression of TrxB was carried out using the optimum expression parameters determined through the above-mentioned expression trials. Purification of the protein from bacterial lysate was performed by immobilized metal affinity chromatography (IMAC) on an ÄKTAprime system using a 1 mL HiTrap Chelating column that was loaded with Ni²⁺ prior to purification.

The His-tagged protein was loaded onto the column and all non-specific bound proteins were washed off. MerA was eluted using a buffer containing an increased concentration of imidazole (20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 7.9). The imidazole was removed by buffer exchange using a 5 mL HiTrap desalting column and a gel filtration buffer (25 mM Tris-HCl, 5 mM MgCl₂, pH 8.0). The protein elution profile was monitored via UV detection at 254 nm. The concentration of TrxB was determined by a Bradford assay to be 2.77 mg/ml.

The large-scale expression of TrxA was carried out using the parameters determined through expression trials (detailed above). The purification of TrxA was conducted according to the same protocol described for TrxB, using both IMAC and gel filtration to obtain a pure protein sample. The concentration of TrxA was determined by a Bradford assay to be 2.43 mg/ml.

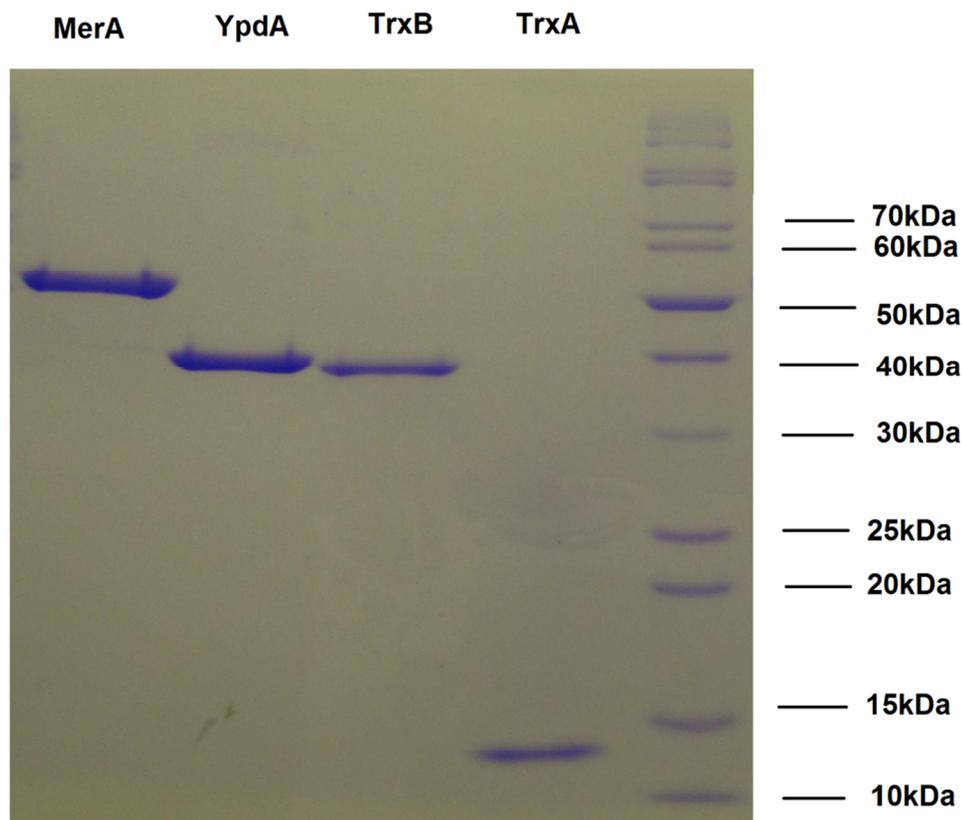


Figure 4.3: SDS-PAGE analysis of soluble TrxB and TrxA protein after IMAC and gel filtration purification. The gel also shows the MerA and YpdA proteins discussed in Chapter 3.

4.2.4 TrxB/TrxA activity assays

4.2.4.1 Confirming TrxB activity and determining its kinetic parameters

Typically, the activity of a TrxR and Trx pair is established in an assay employing insulin as the substrate.^{2, 9} However, pure laboratory-grade bovine insulin could not be obtained for this study due to import constraints, and thus the alternative DTNB-based assay was used to establish TrxB/TrxA activity.

Working in concert, a functional TrxR and Trx are capable of reducing 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) to 5-thio-2-nitrobenzoic acid (NTB²⁻) in a NADPH-dependent reaction.⁷ This reaction is routinely used in activity assays of TrxR-Trx protein pairs.

TrxB and TrxA were demonstrated to be active by observing the NADPH-dependent reduction of DTNB. The kinetic parameters of the reaction were individually determined for TrxA and NADPH, as shown in Figures 4.3A and 4.3B respectively. The assay data was fitted to the Michaelis-Menten equation and kinetic parameters for the reaction were determined as described in Chapter 3. The TrxA protein was compared with Trx1 of *B. subtilis*, each as reaction substrates for *S. aureus* TrxB and *B. subtilis* TR respectively (Table 4.1). TrxA demonstrates comparable affinity towards its TrxR partner, TrxB, as that of Trx1 towards TR.

4.2.4.2 Activity analyses of TrxB and TrxA with CoAD

To determine if TrxB and TrxA are reactive towards CoAD, the enzyme's activity was assayed by substituting DTNB for CoAD as a substrate. Reactions could therefore not be followed based on substrate reduction; instead, the reaction progress was observed spectrophotometrically in a 1 cm pathlength cuvette by monitoring the decrease in NADPH concentration at 340 nm. The resulting activity profile is shown in Figure 4.4. The thioredoxin system's activity towards CoAD was compared to that of CoADR in order to ascertain the system's affinity and efficiency towards CoAD (Table 4.2). The thioredoxin system displays a far lower affinity for CoAD than that of CoADR, with a K_m more than 20-fold higher than CoADR. Similarly, the CoADR-CoAD reaction demonstrates a significantly faster maximal reaction rate and higher turnover over number versus those of the thioredoxin system.

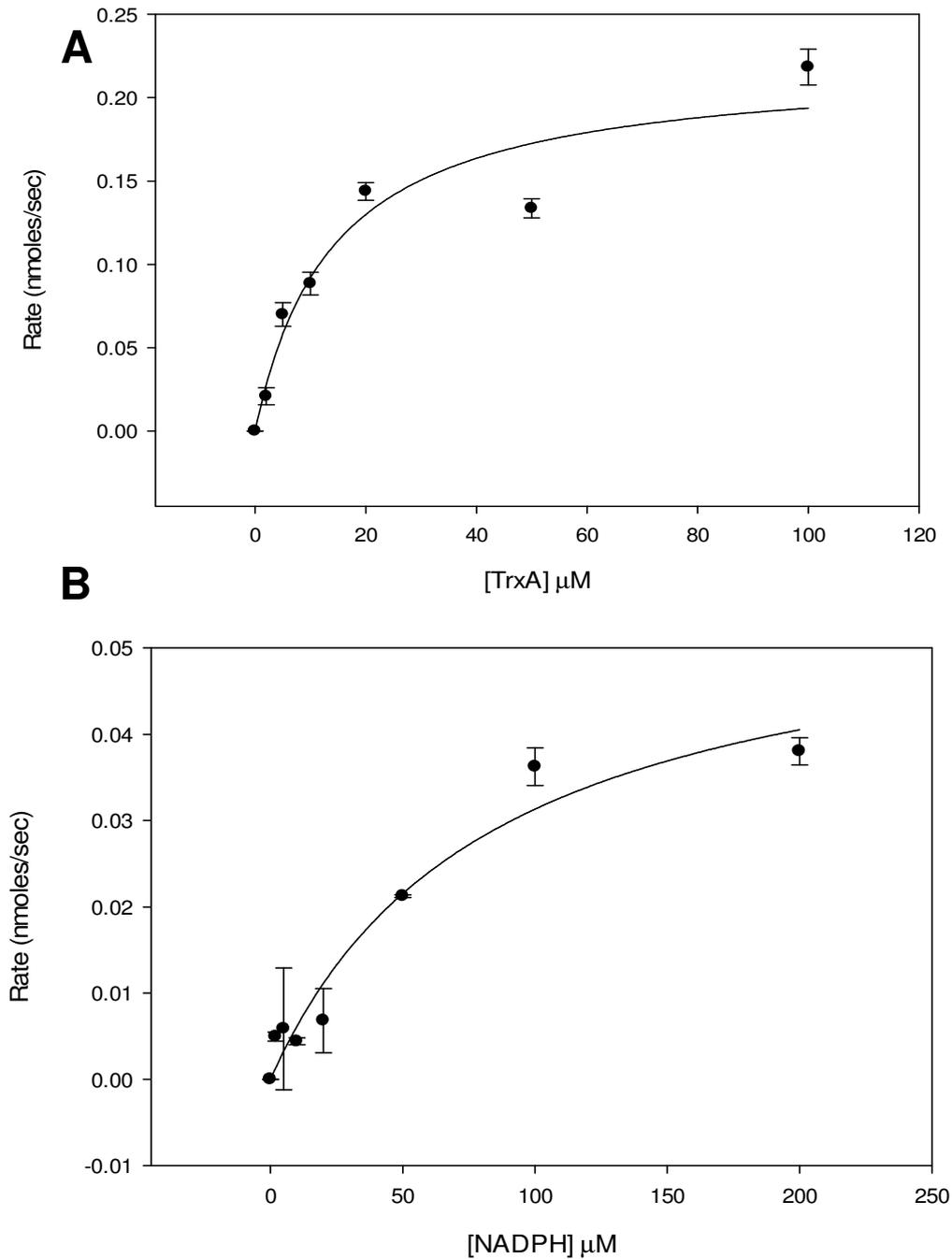
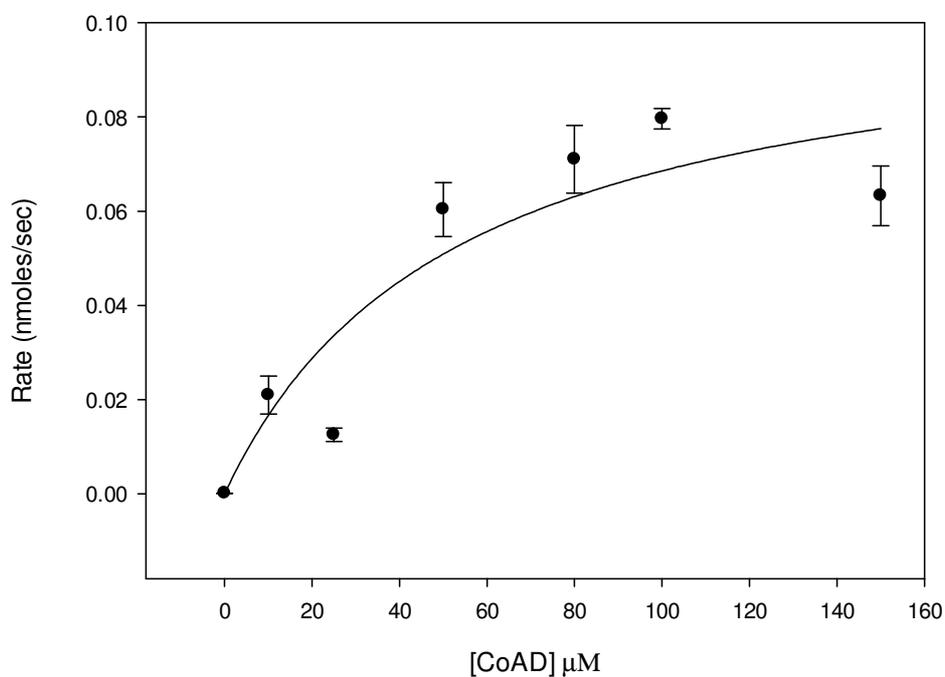


Figure 4.3: Activity of TrxA and TrxB with DTNB. **A)** The concentrations of TrxB, NADPH and DTNB were kept fixed at 15 nM, 200 μM and 1 mM respectively; whilst the TrxA concentration was varied between 0-100 μM . **B)** The concentration of NADPH was varied between 0-200 μM , whilst TrxA, TrxB and DTNB were fixed at 50 μM , 15 nM and 1 mM respectively.

Table 4.1: Comparison of the kinetic parameters of the *S. aureus* thioredoxin system with that of *B. subtilis*.

Substrate	K_M (μM)	V_{max} ($\text{nmols}\cdot\text{s}^{-1}$)	k_{cat} (s^{-1})
TrxA	13.9 ± 3.00	0.221 ± 0.015	73.6
NADPH	83.1 ± 26.2	0.057 ± 0.008	19.1
Trx1 <i>B. subtilis</i> thioredoxin system ⁷	8.41 ± 0.32	-	13.5 ± 0.15

**Figure 4.4:** Activity of the thioredoxin system towards CoAD. CoAD concentration was varied between 0-150 μM .**Table 4.2:** The kinetic parameters of the thioredoxin system and CoADR with CoAD

Substrate Enzyme(s)	K_M (μM)	V_{max} ($\text{nmols}\cdot\text{s}^{-1}$)	k_{cat} (s^{-1})
CoAD TrxB & TrxA	53.0 ± 22.7	0.105 ± 0.02	0.327
CoAD CoADR	2.36 ± 0.32	0.011 ± 0.00	2.75

4.2.4.3. Activity analyses of TrxB and TrxA with pantethine

Following the poor results of the thioredoxin system with CoAD, pantethine was selected for testing. The compound, which is a CoA precursor and breakdown product, is both smaller and less negatively charged than CoA. These features may play a role in the thioredoxin system's ability to reduce a particular disulfide substrate, and pantethine was thus assayed in order to test this. TrxB and TrxA were assayed with pantethine under the conditions used for the activity test with DTNB.

The reaction progress was observed spectrophotometrically in a 1cm pathlength cuvette by monitoring the NADPH decrease at 340nm. Kinetic parameters were determined for the reaction according to the procedure detailed in Chapter 3, with the Michaelis-Menten plot shown in Figure 4.5. The kinetic parameters for the thioredoxin system-pantethine reaction are shown in Table 4.3, as well as compared to those of the other LMW thiol disulfide substrates in Table 4.5. The results show that the thioredoxin system does not reduce CoAD as well as CoADR, but that this poorer reaction is not due to the charge or size of CoAD.

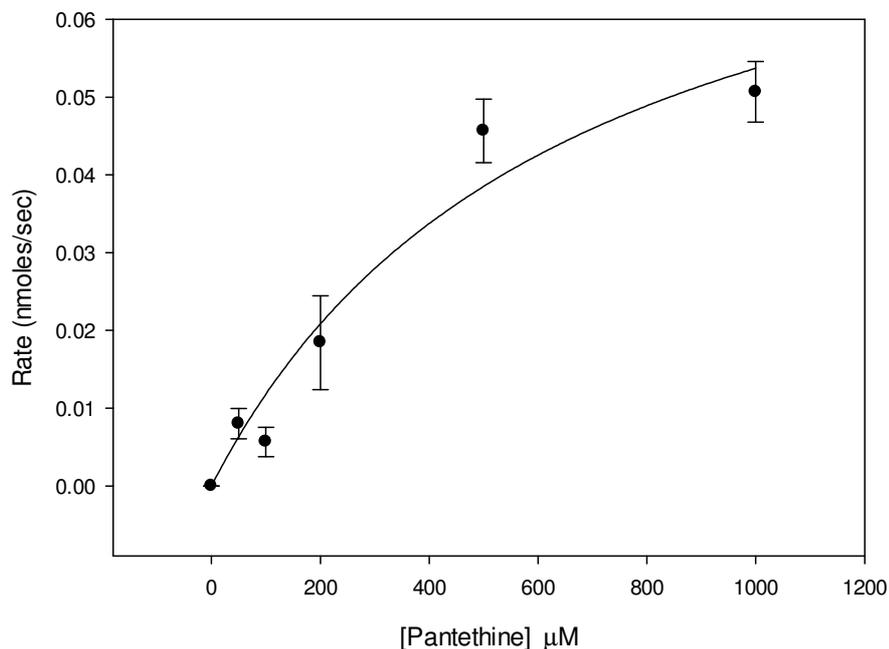


Figure 4.5: Activity of the thioredoxin system towards pantethine. The pantethine concentration was varied between 0-1000 μM.

Table 4.3: The kinetic parameters of the thioredoxin system towards the LMW thiol disulfide, pantethine

Substrate	K_M (μM)	V_{max} ($\text{nmols}\cdot\text{s}^{-1}$)	k_{cat} (s^{-1})
Pantethine	650 ± 209	0.089 ± 0.015	0.277

4.2.4.4 Activity analyses of TrxB and TrxA with BSSB

The results of Chapter 3 rule MerA and YpdA out for displaying any direct activity towards BSSB. Thus, the potential activity of the thioredoxin system towards this particular LMW disulfide was investigated.

TrxB and TrxA were assayed with BSSB under the conditions used for the activity test with DTNB; reaction progress was observed by monitoring the NADPH decrease at 340nm. The kinetic parameters for the reaction were determined as described in Chapter 3, with the Michaelis-Menten plot shown below (Figure 4.6). The calculated kinetic parameters are compared to those of the *B. subtilis* thioredoxin system in Table 4.4, as well as the other LMW disulfide substrates in Table 4.5.

The *S. aureus* thioredoxin system shows very similar affinity towards BSSB as that of *B. subtilis*, but has a turnover number that is nearly 20-fold higher.

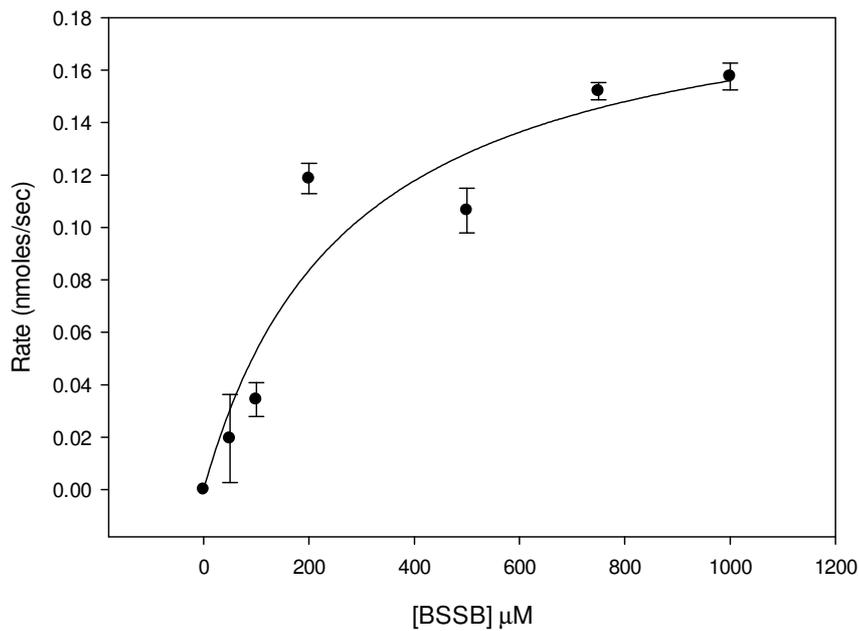


Figure 4.6: Activity of the thioredoxin system towards BSSB. BSSB concentration was varied between 0-1000 μM . The kinetic parameters obtained for the *S. aureus* thioredoxin system with BSSB are compared to that of the *M. tuberculosis* system, as described by the Hamilton group.[‡]

Table 4.4: The kinetic parameters of the thioredoxin system towards the LMW thiol disulfide, BSSB.

Substrate Enzyme	K_M (μM)	V_{max} (nmols. s^{-1})	k_{cat} (s^{-1})
BSSB <i>S. aureus</i> TrxB/TrxA	275 ± 80.8	0.199 ± 0.021	0.621
BSSB <i>M. tuberculosis</i> TrxB/TrxC [‡]	291 ± 64	-	0.0314

[‡]Unpublished results, C.J. Hamilton et al. (University of East Anglia) (Personal communication).

4.2.4.5 Comparison of the TrxB/TrxA kinetic parameters with different substrates

The kinetic parameters of the thioredoxin system with the three different disulfides that were tested are compared in Table 4.5. Of the three substrates assayed, CoAD appears to be the favoured substrate. The TrxA protein displays the highest affinity for CoAD, with a K_M 5-fold and 12-fold lower than that of BSSB and pantethine respectively. Additionally, the catalytic efficiency calculated for CoAD is the highest of the three substrates, providing a basis for *S. aureus*'s ability to survive even in the absence of a functional CoADR enzyme.

Table 4.5: A comparison of the kinetic parameters of the thioredoxin system with different disulfide substrates.

Substrate	K_M (μM)	V_{max} (nmoles. s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{s}^{-1}.\text{mM}^{-1}$)
CoAD	53.0 ± 22.7	0.105 ± 0.02	0.327	6.17
BSSB	275 ± 80.8	0.199 ± 0.021	0.621	2.25
Pantethine	650 ± 209	0.088 ± 0.015	0.276	0.426

4.3 Discussion

A 1992 study by Aharonowitz *et al.* identified a putative thioredoxin system in *Streptomyces clavuligerus* that showed disulfide reductase activity towards CoAD.¹ Aside from this study, a review of the current literature on bacterial thioredoxin systems indicates that any activity between the system and CoAD remains untested. As CoA appears to be the predominant LMW thiol active in maintaining *S. aureus* redox balance, the non-essentiality of the CoADR protein indicates that a secondary system must exist to fulfil this activity if needed.⁴ Verifying the existence of such a system and its activity was thus essential.

The effective and reproducible reduction of CoAD by the *S. aureus* thioredoxin system indicates that the compound is a viable substrate for the system. TrxB and TrxA thus appear capable of acting as an effective back-up system to CoADR, reducing CoAD during oxidative stress and/or under conditions when CoADR is not fully functional.

TrxB/TrxA's activity toward pantethine, the disulfide of pantetheine, was also tested. Pantetheine is a breakdown product of CoA, and also serves as the precursor to CoA salvage biosynthesis.⁵ Pantethine may also be broken down in the cell to form pantothenic acid and cysteamine, after which the former is phosphorylated as part of the first step in the

synthesis of CoA.⁵ Pantethine was tested as a substrate of TrxB /TrxA based on its importance as an alternative CoA precursor and breakdown product, and also to ascertain whether the system shows great activity to one of CoA's moieties which is uncharged. However, the thioredoxin system enzymes did not display high affinity towards pantethine however, and the overall catalytic efficiency of the reaction was low compared to that of CoAD.

Although numerous proteins have been reported to be involved in BSH-related pathways,^{6, 8} a dedicated BSSB-reductase protein or system has yet to be experimentally verified. The results presented in Chapter 3 demonstrated that neither MerA nor YpdA had any BSSB reductase activity. A study of the *B. subtilis* thioredoxin system by the Hamilton lab at the University of East Anglia demonstrated that it is capable of reducing BSSB effectively. Therefore the *S. aureus* TrxB/TrxA system's ability to reduce BSSB was tested, and found to reduce BSSB equally well, with comparable kinetic parameters to those reported by Hamilton *et al.* for the *B. subtilis* proteins. Consequently, the thioredoxin system remains the only protein system with demonstrable BSSB-reducing activity in BSH-containing bacteria.

4.4 Conclusion

The thioredoxin system of *S. aureus* is a versatile system capable of reducing multiple disulfide substrates, including LMW disulfides. In this study was demonstrated that TrxB/TrxA is capable of reducing the disulfides of both CoA and BSH, the LMW thiols of *S. aureus* known to play a role in the maintenance of redox balance and xenobiotic detoxification. Additionally, the thioredoxin system is able to reduce the compound pantethine which is directly involved in the metabolism of CoA. Therefore, the thioredoxin system of *S. aureus* exhibits much substrate promiscuity, which allows it to play a critical role in the maintenance of *S. aureus* redox balance beyond its usual roles associated with the reduction of essential proteins. These findings provide additional support for TrxB as a viable target for the development of novel antibiotics.

4.5 Experimental

4.5.1 Materials

All buffer components were purchased from Sigma-Aldrich. BSSB was generously provided by the Hamilton group of the University of East Anglia. CoAD was synthesised by Bertus Moolman at the University of Stellenbosch. Restriction enzymes were purchased from Fermentas. All primers used were synthesised by Inqaba Biotec in Pretoria.

4.5.2 DNA amplification

4.5.2.1 *trxB*

The *trxB* gene (SAOUHSC_00785) was amplified from genomic *Staphylococcus aureus* DNA strain RN4220 (isolated using the DNA extraction kit from Novagen) using the following primers: Forward: 5'-GGAGGCGTTAATCATATG ACTGAAATAGAT-3' and reverse: 5'-TCTTAATTCGACCTCGAGTTAAGCTTGATCGTT-3'. With these primers an NdeI (underlined) and an XhoI (underlined) restriction enzyme site is introduced in the forward primer and the reverse primer respectively. Amplification of the *trxB* gene was performed as follows: Tubes 1 to 3 contained Pfu buffer, 0.4 mM dNTP mix, 1 mM forward primer, 1 mM reverse primer, 1 ng genomic DNA, 1.25 U Pfu DNA polymerase and distilled, deionized water (ddH₂O) to a final volume of 25 μ L. The PCR program had an initial step of 2 min at 94°C followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s and polymerization for 1 min at 70°C. Following amplification, the reaction mixtures were visualised and the product band purified as previously outlined. Excellent results were obtained at MgSO₄ concentrations of both 3 mM and 4 mM.

4.5.2.2 *trxA*

The *trxA* gene (SAOUHSC_01100) was amplified from genomic *Staphylococcus aureus* DNA strain RN4220 using the following primers: Forward: 5'-GGATTGGCACATATG GCAATCGTAAAA-3' and reverse: 5'-GTCGTCATTGGTCTCGAGTTATAAATGTTTATC-3'. With these primers an NdeI (underlined) and an XhoI (underlined) restriction enzyme site is introduced in the forward primer and the reverse primer respectively. Amplification of the *trxA* gene was performed according to the same procedure described for *trxB*. Following amplification, the reaction mixtures were visualised and the product band purified as previously outlined. Excellent results were obtained at MgSO₄ concentrations of both 3 mM and 4 mM.

4.5.3 Restriction digests

Restriction digests were carried out on both the *trxB* and *trxA* PCR products, as well as pET28a(+), using NdeI and XhoI restriction enzymes (Fermentas). The pET28a(+) plasmids were isolated from *E. coli* using the Zippy® plasmid preparation kit (Fermentas). The digestions were performed using Fermentas FastDigest buffer, 16 μ L PCR product/plasmid and 10U each NcoI and XhoI. These reactions were incubated at 37°C for 1 hour prior to 1%

agarose gel electrophoresis. Bands of the correct size were excised and purified using the Novagen gel clean-up system.

4.5.4 Plasmid construction

The pET28a(+) plasmid system was used for the construction of all expression vectors required for this study. The molar concentrations of both the gene inserts and isolated plasmids were obtained using the Qubit® Fluorometric Quantitation system (Life Technologies). Equal molar amounts of the restriction digested plasmid and PCR product were added together in a volume of 5 µL. A final volume of 10.5 µL was obtained by adding Quick Ligation Buffer and 10 U of Quick T4 DNA Ligase (both purchased from New England Biolabs) to the reaction mixture. Following incubation for 5 minutes at room temperature, the mixture was transformed into competent *E. coli* Mach1 cells. Transformation was performed as follows: 1 µL of the plasmid-insert construct was added to 80 µL of chemically-competent Mach 1 cells, and incubated on ice for 30 mins. The cell mixture was heat-shocked at 42°C for 45 seconds, and then cooled on ice for 5 mins. The cell mix was then incubated at 37°C for 1 hour before being centrifuged at 8000 RPM for 4 mins. Half of the supernatant was discarded, with the cell pellet being re-suspended in the remaining supernatant. The re-suspended cells were then plated onto LB plates containing 30 mg/L kanamycin and incubated overnight at 37°C.

Colonies were screened for the *trxA* and *trxB* gene inserts via electrophoresis on a pre-stained 1% agarose gel, using the parent pET28a(+) plasmid as a reference. Only plasmids that appeared larger than the pET28a(+) were selected for further screening. The corresponding colonies of the chosen plasmids were cultured overnight in 30 mg/L kanamycin LB media at 37°C. Plasmids from these cultures were purified using the Zippy Plasmid Miniprep I Kit (Zymo Research) and subjected to a restriction digest reaction with the appropriate restriction enzymes. The restriction digestion reactions were subsequently visualised on a pre-stained 1% agarose gel and, if the correct band pattern was observed, the successfully constructed plasmids were stocked for future use.

4.5.5 Protein expression and purification

Protein expression for all targets was first conducted in small scale in order to determine optimum conditions for soluble expression of the proteins of interest. Following determination of these conditions, large scale expression of target proteins was performed. All growth media and plates used in the expression of the target proteins contained the necessary concentrations of the antibiotic kanamycin (30 mg/L).

4.5.5.1 Expression trials

An expression plasmid was selected from those constructed earlier and transformed into *E. coli* BL21*(DE3) cells. An overnight culture was grown from a single colony of the transformed cells in preparation for expression trials. All expression trials were conducted in 15 mL Corning® centrifuge tubes. Each tube contained 4 mL LB media and 100 µL overnight culture, forming an expression culture. Expression cultures were incubated at 37°C with shaking until an OD₆₀₀ reading of 0.6 was reached, at which point IPTG was added from a 10 mM stock. Following the addition of IPTG, the cultures were topped up with LB media to give a final volume of 5 mL. The following parameters were varied in order to determine the optimum environment for soluble protein expression:

- The final IPTG concentration in each tube: 1mM, 0.5 mM, 0.2 mM and 0.05 mM.
- The temperature at which expression took place: 22°C or 37°C.

Following overnight expression, the cells were harvested via centrifugation and either processed immediately or stored at -20°C for use at a later opportunity.

4.5.5.2 Large scale expression

LB media (500 mL) was inoculated with 2 mL of an overnight culture of *E. coli* BL21*(DE3) containing the desired expression plasmid. The culture was incubated on an orbital shaker at 37°C until an OD₆₀₀ of 0.5-0.6 was reached. The optimum amount of IPTG (pre-determined from small scale trials) was added and the culture was further incubated at the similarly determined temperature overnight. Cells were then collected through centrifugation at 4500×g for 30 minutes at 4°C. As with small scale expression, the pellets obtained were either processed directly after collection or stored at -20°C until required.

4.5.5.3 Analysis of expression trials

Cell pellets obtained from small scale expression trials were re-suspended in 300 µL BugBuster® protein extraction solution (Novagen) and incubated on a shaking platform for 20 minutes at room temperature. The reaction mixture was centrifuged at 1300×g for 20 minutes, with the supernatant subsequently transferred to a clean tube. The cell debris pellet was re-suspended in 100 µL of 10 mM Tris-HCl, pH 8.0. 15 µL of both the supernatant and re-suspended cell pellets were analysed on a 12% SDS-PAGE gel for soluble and insoluble proteins respectively.

4.5.5.4 Purification of large scale protein expression

The cell pellets obtained from large scale expression were re-suspended in binding buffer (20 mM Tris-HCl, 5 mM imidazole, 500 mM NaCl, pH 7.0), in a volume equating to 10 mL per 1 g of pellet. Cells were lysed via sonication, and the cell debris removed by centrifugation at 1500×g for 30 minutes at 4°C. The supernatant was filtered with a Pall Acrodisc PSF GxP/GHP 0.45 µm filter prior to injection into the ÄKTApriime system (Amersham Biosciences).

All proteins were purified using a 1 ml HiTrap Chelating HP column. Prior to loading the protein, the column was equilibrated with 10 column volumes of H₂O and 5 column volumes of binding buffer (20 mM Tris-HCl, 5 mM imidazole, 500 mM NaCl, pH 7). The protein was loaded and followed by two wash steps: 10 column volumes of binding buffer, followed by another 5 column volumes containing 15% elution buffer (20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 7.9). This second wash step was essential for eliminating any non-specifically bound proteins. The recombinant TrxB-His and TrxA-His proteins were eluted over the course of 10 column volumes of 100% elution buffer. For the duration of the purification, the flow rate was maintained at 1ml/min, whilst protein elution profiles were monitored at 254 nm. All columns used were purchased from Amersham Biosciences.

4.5.5.5 SDS-PAGE gel analysis

Proteins obtained from both small and large scale expression were visualised by SDS-PAGE using 12% gels. An equal volume of 2× SDS-PAGE loading buffer (0.125 M Tris-HCl, 4% SDS, 30% glycerol, 1.5% β-mercaptoethanol and bromophenol blue, pH 6.8) was added to the protein samples and the reaction mixtures were incubated at 95°C for 5 minutes. 15 µL of the samples were loaded onto the gel and electrophoresed in SDS-PAGE running buffer (0.1% SDS, 0.025 M Tris and 0.192 M glycine). Completed gels were stained with Coomassie blue stain (45 mL MeOH, 40 mL ddH₂O, 10 mL acetic acid and 250 mg Coomassie Brilliant Blue) for 30 minutes and later de-stained for 2 hours using destain solution (45 mL MeOH, 40 mL ddH₂O, 10 mL acetic acid).

4.5.5.6 Protein concentration determination

All protein concentration was determined using the Bradford protein assay in 96-well plates. A standard curve of absorbance versus concentration was plotted with the use of BSA standards (Biorad). All protein samples and standards were treated with Coomassie Brilliant

Blue and incubated at room temperature for 5-10 minutes. Absorbance was subsequently measured at 595 nm using a Thermo Varioskan multiplate spectrometer.

4.5.6 Enzymology

Unless otherwise stated, all assays performed were done in triplicate.

4.5.6.1 TrxB and TrxA activity assay: Trx K_m

The assay conditions were adapted from protocols set out by Aharonowitz et al. and Gustafsson *et al.*^{2, 7} A master mix was prepared containing 0.26 M HEPES (pH 7.6), 0.01 M EDTA, 2.0 mM NADPH and 15nM TrxB, whilst TrxA concentrations were varied between 2-100 μ M. Solutions of 1 mM DTNB were prepared, and all reagents were incubated at 37°C for 10 min prior to commencing. The reaction was initiated by adding 160 μ L of master mix to 40 μ L DTNB in a 96-well plate. The increase in absorbance at 412 nm was followed for 2 min at 37°C in a Thermo Varioskan multiplate spectrometer. All assays were performed in triplicate.

4.5.6.2 TrxB and TrxA activity assay: NADPH K_m

Assay adapted from protocols set out by Aharonowitz et al. and Gustafsson *et al.*^{2, 7} A master mix was prepared containing 0.26 M HEPES (pH 7.6), 0.01 M EDTA, 2.0 mM, 15nM TrxB and 50 μ M TrxA. NADPH concentrations were varied between 2-200 μ M. Substrate solutions of 1 mM DTNB were prepared, and all reagents were incubated at 37°C for 10 min prior to commencing. The reaction was initiated by adding 160 μ L of master mix to 40 μ L DTNB in a 96-well plate. The increase in absorbance at 412 nm was followed for 2 min at 37°C in a Thermo Varioskan multiplate spectrometer. All assays were performed in triplicate.

4.5.6.3 CoAD assay

Assay adapted from protocols set out by Aharonowitz et al.² A pre-mix was prepared containing 0.26 M HEPES (pH 7.6), 0.01 M EDTA and 2.0 mM NADPH. The master mix was prepared by adding 40 μ L of pre-mix to 40 μ L of undiluted TrxB protein and 20 μ L of undiluted TrxA enzyme. Substrate solutions of CoAD were prepared, with the concentrations varied between 0-100 μ M. Both the master mix and substrate mixes were incubated at 37°C prior to the assay. The reaction was initiated through the addition of 100 μ L of master mix to 20 μ L of substrate, mixed briefly, and 120 μ L of the reaction mixture transferred to a 1 cm

pathlength cuvette. The decrease in absorbance at 340 nm was followed for 2 min at 37°C in an Agilent Cary 60 UV-Vis spectrometer. All assays were performed in triplicate.

The above procedure was repeated for the assays with BSSB and pantethine as substrates. For both the BSSB and pantethine assays, the substrate concentrations were varied from 0-1000 µM.

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Chapter 5

Conclusions and future work

5.1 Summary of results achieved

This study set out to functionally characterize four proteins implicated in maintaining the redox balance of *S. aureus*. Two FDRs, MerA and YpdA, were shown to not participate in the direct reduction of LMW thiol disulfides. MerA instead demonstrated the ability to reduce two biologically-relevant transition metal ions without the aid of an interacting protein partner: Hg^{2+} and Fe^{3+} . Fe^{3+} was further shown to be favoured over Hg^{2+} as a substrate of MerA, which has implications for the protein's role in oxidative stress resistance.

YpdA was shown to be incapable of reducing any of the LMW thiol disulfides found in *S. aureus*, even in the presence of a generic TFP protein, TrxA. This suggests that YpdA may be unable to perform catalytic functions in the absence of its cognate interacting partner. Alternatively, the protein may perform a different cellular function unrelated to LMW disulfide reduction or oxidative stress resistance.

The thioredoxin system proteins of *S. aureus*, TrxB and TrxA, demonstrated measurable reduction of the three LMW thiol disulfides assayed. However, the kinetic parameters determined for the reactions suggest that the thioredoxin proteins do not display great affinity for these substrates. Therefore, these proteins may be more likely to reduce LMW thiol disulfides under critical conditions, as opposed to acting as the primary reducing system for these compounds.

5.2 Assigning oxidative stress-related functions to MerA and YpdA

In Chapter 3, the attempts at assigning substrates and catalytic function to MerA and YpdA are detailed. Sequence comparison of the MerA protein to that of known mercuric reductases revealed two missing components: both the N- and C-terminal Cys-pairs. MerA was however shown to possess more fundamental features, such as the core, catalytic site Cys pair, and intact NADPH- and FAD-binding domains.

Demonstrating LMW thiol disulfide reductase activity in MerA was unsuccessful, as the protein proved incapable of reducing CoAD, BSSB or pantethine. Thereafter, MerA was tested for its ability to function as a mercuric reductase. Although successful, the protein

showed a greatly decreased affinity for HgCl_2 compared to that of known mercuric ion reductases.² This has been attributed to MerA's lack of C-terminal Cys-pair, a feature which has been suggested to be involved in Hg^{2+} acquisition.¹ Subsequent tests with MerA and Fe^{3+} revealed that the protein was capable of reducing the metal ion. Moreover, MerA displayed affinity for Fe^{3+} comparable to that of known ferric ion reductases from other bacterial species.⁶

These results indicate that MerA preferentially reduces Fe^{3+} as a substrate. Since the protein has been strongly implicated in oxidative stress resistance functions,⁷ this study proposes a role for MerA in the reactivation of oxidised 4[Fe-S] cluster proteins by providing Fe^{2+} .

A sequence comparison of YpdA to that of a known TrxR protein showed a fundamental disparity between the proteins: YpdA lacked the active site CxxC motif associated with thioredoxin reductase function.⁴ Additionally, the protein displayed a four residue insert within the NADPH-binding domain, suggesting that it may be unable to bind the cofactor. Although anaerobic titrations of YpdA with NADPH disproved any difficulty binding the cofactor, the missing active site Cys pair gave pause to the protein's potential for disulfide reduction activity.

Assaying YpdA with the LMW thiols CoAD, BSSB and pantethine yielded no measurable disulfide reductase activity. Concern that the protein may be unable to function without an interacting partner lead to a new strategy: providing YpdA with a generic Trx partner, in the form of the characterised TrxA. Under these conditions, no reduction of the thiol disulfides was detected.

Based on the results, this study can conclude that YpdA is not a direct BSSB reductase, as suggested by several studies.^{3, 5} However, the protein may interact with one of the proposed "bacilliredoxins" to reduce BSSB in a manner resembling that of the Grx/GSH/GR system.⁵ Further work will therefore be necessary in order to draw a final conclusion on the potential activity of YpdA.

5.3 Assessing the activity of the *S. aureus* thioredoxin system towards LMW thiol disulfides

In Chapter 4, the TrxB and TrxA proteins were assayed with disulfides of LMW thiols known to be involved in *S. aureus* redox balance homeostasis, namely CoA and BSH. The thioredoxin system was shown to successfully reduce the disulfide forms of both compounds. For CoAD in particular, the proteins demonstrated a much lower affinity for the compound than that of CoADR, indicating that they are not primarily involved in such a

reaction. Additionally, the thioredoxin system was found to reduce the compound pantethine, a metabolic precursor and breakdown product of CoA. This study thus concludes that the thioredoxin system of *S. aureus* demonstrates much substrate promiscuity. Such promiscuity affords the system a role in the maintenance of *S. aureus* redox balance beyond its usual roles associated with the reduction of essential proteins. Therefore, TrxB and TrxA may be able to function as a back-up system for redox balance in critical conditions. Together, these findings further highlight TrxB as a viable and important target for the development of novel antibiotics.

5.4 Future work

5.4.1 The role of MerA in 4[Fe-S] cluster protein reactivation

The proposal that MerA may provide Fe^{2+} for the reactivation of 4[Fe-S] cluster proteins must be investigated. Future studies on this topic will involve the development of *merA*-null *S. aureus* mutants that may be subjected to oxidative stress, after which the 4[Fe-S] protein integrity may be assessed. Additionally, pull-down assay studies with the MerA protein will be conducted in order to establish any protein-protein interactions MerA may have in the cell. MerA's iron reducing abilities will also be further tested with other Fe (III) compounds, such as Fe (III) citrate.

5.4.2 The interacting partners of YpdA and its role with BSSB

The proposed bacilliredoxins, YphP and YtxJ, will be cloned and purified for assays with YpdA. Basic assays with BSSB as a substrate will be conducted, providing YpdA with the putative bacilliredoxins as interacting partners. Additional FRET experiments may be conducted to assess any interaction between YpdA and the putative bacilliredoxins. Failing noticeable interaction, YpdA may be used as a bait protein in pull-down assays to identify any protein partners.

5.5 References

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